VACCINES CONTAINING RECOMBINANT PILIN AGAINST
NEISSERIA GONORRHOEAE OR *NEISSERIA MENINGITIDIS*

Field of the Invention

This invention relates to the use of
recombinant pilin proteins in vaccines to protect
against disease caused by *Neisseria gonorrhoeae* or
Neisseria meningitidis.

Background of the Invention

Neisseria gonorrhoeae (*N. gonorrhoeae*) and
Neisseria meningitidis (*N. meningitidis*) are Gram-
negative cocci. *N. gonorrhoeae* and *N. meningitidis* are
genetically very closely related, but the clinical
manifestations of the diseases they produce are very
different. *N. gonorrhoeae* causes gonorrhea, while *N.
meningitidis* causes meningococcal meningitis. These
bacteria of the genus *Neisseria* inhabit mucosal
surfaces of the body.

Type IV pili are nonflagellar hairlike
structures on the surface of numerous Gram-negative
bacteria, including *Dichelobacter* (formerly
Bacteroides) *nodous*, *Eikenella corrodens*, *Kingella
denitrificans*, *Moraxella bovis*, *M. lacunata*, *M.
nonliquefaciens*, *N. gonorrhoeae*, *N. meningitidis*, and
Pseudomonas aeruginosa (Bibliography Entries 1,2). The
toxin co-regulated pili from *Vibrio cholerae* and the
bundle forming pili of enteropathogenic *Escherchia coli*
exhibit a limited number of similarities to type IV
pili and are considered to be more distantly related
(1,2). For both *N. gonorrhoeae* and *N. meningitidis*,
piliated bacteria adhere to a variety of epithelial
cells of human origin much more avidly than do

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nonpiliated cells, and thus the pili are thought to act as virulence factors by anchoring the organisms to mucosal infection sites.

Type IV pili are 5-7 nM in width and up to 5 μM in length. The pilin protein subunits are linked in tandem to form long, thin polymers. In the case of the pathogenic *Neisseria*, the pili are apparently homopolymeric in nature, being comprised of a single structural subunit, the pilin protein. The pilin protein has a molecular weight of 13,000 to 22,000 daltons (1,2,3).

In nature, the pilin protein is assembled into a helical structure called a pilus (plural: pili) at the bacterial outer membrane that has a molecular weight of approximately 10^7 daltons. When purified pili are dialyzed against pH 12 phosphate buffer, the intact pili are irreversibly dissociated into aggregates of the pilin protein which are called pilin oligomers (4). These pilin oligomer aggregates (molecular weight of approximately 600,000 daltons) are much smaller in size than the intact pilus.

N. gonorrhoeae expresses a single pilin protein which was first isolated and sequenced by Schoolnik and co-workers (5). The gonococcal pilin protein consists of three regions: (a) the highly conserved amino terminal region (residues 1-53); (b) the middle third (residues 54-124) which exhibits a limited amount of sequence variation and (c) the carboxy third of the protein (residues 125-160) which contains a highly variable disulfide loop.

During the course of natural infections, pili undergo high frequency phase and antigenic variation (3,6). The genetics of this variation are extraordinarily complex and have been extensively studied. Each strain of *Neisseria gonorrhoeae* has the

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ability to change the primary amino acid sequence of
the pilin molecule, and thus the antigenic nature of
its pili. The molecular mechanism responsible for this
variation involves a nonreciprocal recombination event
5   between the expression locus (*pil*E) and numerous (17 to
19), promoterless, silent (*pilS*) genes (6). Pilin
sequences (or portions thereof) move from the *pilS* loci
into an expression locus to generate new pilin
variants, which in turn enables gonococci to express an
10   extremely large number of pilin proteins.
          In contrast to *N. gonorrhoeae*, *N.*
meningitidis expresses two distinct classes of pilin
named class I and class II. As in the gonococci, the
meningococcal class I pili undergo antigenic and phase
15   variation (3). Class I pilins have been shown to be
similar to gonococcal pilin in terms of molecular
weight (17-20 kd) and reactivity with a monoclonal
antibody (SM1) which binds to a highly conserved
epitope on the gonococcal pilin (3). A number of
20   class I pilins have been cloned and the amino acid
sequences have been shown to have a high degree of
similarity to the sequence of the gonococcal pilin (7).
In contrast, class II pilins do not react with the SM1
antibody and have a lower molecular weight (13-16 kd).
25   Several strains expressing class II pilin have been
shown to react with a polyclonal antisera directed
against gonococcal pili (3). Aho et al. (7) have
recently determined the sequence of a class II pilin.
The first third of the neisserial pilins are
30   essentially identical. The class II pilin protein
differs from the class I pilin and gonococcal pilin
proteins in the hypervariable region of the protein
where a large deletion has occurred. Achtman et al.
(8), using monoclonal antibodies specific for class I
35   or class II pilins, demonstrated that some serogroup A

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isolates from Africa bound both antibodies. It has
been demonstrated that strains expressing class II pili
also have truncated, silent class I pilin genes (7).
Taken together, these data suggest the possibility that
a single meningococcal cell might express both pilin
proteins simultaneously.

Because pili are believed to mediate the
initial contact with mucosal cells, there has been
considerable interest in using these structures as
vaccine antigens to prevent disease caused by piliated
bacteria. Pili vaccines against traveler's diarrhea
and gonorrhea have been tested in human beings (9).
However, to date, they have been efficacious only
against homologous strains. A number of pili-based
vaccines have been reported for diseases affecting
domestic livestock such as infectious
keratoconjunctivitis (pinkeye) in cattle (1), footrot
in sheep (1,10) and diarrhea in piglets (9) or calves
(9). In each of these veterinary examples, the pili
vaccine provided protection against challenge by
strains expressing the homologous, but not
heterologous, pili.

The earliest gonococcal vaccines contained
whole organisms and provided little or no protection
(11). Recent vaccine development against gonorrhea has
focused on purified surface components, in particular,
pili (9,11) and the porin protein (P.I or Por) (11).
To date, however, only pili have been shown to protect
humans from challenge, and this was limited to
protection against the homologous strain (12). A
denatured form of gonococcal pili (4) has been shown to
generate antibodies in mice or guinea pigs which bind
to heterologous pili *in vitro*. However, this has not
been considered a commercially viable approach because
of the difficulty in growing piliated gonococci in

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liquid media (a necessity for commercial production)
(1). Based on the success of the initial human
challenge studies, the gonococcal pilus vaccine was
tested in a large, placebo-controlled double blind
5   efficacy trial (12). In this trial, the vaccine failed
to protect male volunteers from gonococcal infections.
It was postulated that the most likely explanation for
this was pilus heterogeneity (12).

       Indeed, the antigenic variability of the
10   pilin proteins, both gonococcal and meningococcal, has
been repeatedly cited as a major obstacle in the
development of pili-based vaccines (3,13,14,15,16,17).
It has been suggested that the dominant immune epitope
on the assembled, intact pilus is the disulfide loop,
15   which exhibits the greatest sequence variation (17,18).
This may account for the failure of the Korean field
trial with formalin-treated intact pili from N.
gonorrhoeae strain Pgh3-2 (12). In addition, the
literature contains a number of references in which
20   antisera directed against purified pili, or pilin
fragments, bound to denatured (Western blot) or
isolated neisserial pili, but did not bind to
heterologous pili on bacterial surfaces (16,17,19,20).
Whether this is due to antigenic variation or
25   concealment of the epitopes in the assembled pilus has
not been completely resolved. This is reinforced by
several reports which demonstrated that the only
monoclonal antibodies exhibiting functional activity in
vitro were those which did not bind to heterologous
30   pili (3). It has been shown for the intact pili from
M. bovis (21) and D. nodosus that a protective immune
response elicited by pili is possible (10). These
vaccines, however, were only able to protect against
challenge with bacteria expressing the homologous pili
35   -- not heterologous pili (10,21). The consensus of the

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scientific community appears to be that pili-based
vaccines, if possible, will protect only against
bacteria expressing the homologous pili.

Recombinant expression of assembled pili has
5   been described for a number of organisms, and is
dependent on the presence of appropriate transport and
assembly genes (22). In *Neisseria*, the genes encoding
the proteins involved in pili assembly and export are
not found in a single contiguous operon, so this
10   approach was not feasible. Another alternative chosen
in European Patent 202,260 B1 was to express type IV
pilin genes in a bacterial host which already possessed
the proteins required for assembly of a different type
IV pilus; e.g., *Pseudomonas aeruginosa* (23). But as
15   reported by Hoyne et al., the expression and assembly
of gonococcal pili at the outer membrane of *Pseudomonas*
was unstable (24). When the recombinant strain was
grown in liquid media in the presence of selective
antibiotics, it was overgrown by wild-type, piliated
20   *Pseudomonas*. The authors stated:

"[T]he compatibility of foreign mePhe [N-
methylphenylalanine] pilus production in host
strains will depend on the extent of divergence of
host and donor pilus assembly systems. The
25   observed instability of PAK/2PfS [*Pseudomonas
aeruginosa* strain K/2PfS] expressing gonococcal
pilin ... may indicate that the limits of
interspecies expression of mePhe pili are being
approached in this instance." (24)

30   Because of this result, the expression of gonococcal
pili in *Pseudomonas* was not viewed as a commercially
viable approach.

Elleman, Egerton and co-workers described the
development of a vaccine against ovine footrot using
35   intact pili from *Dichelobacter nodosus*. Field trials

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had demonstrated that intact pili protected against *D. nodosus* strains expressing the homologous pili. This meant that a commercial vaccine would need to contain eight or nine different pili in order to achieve comprehensive protection. In an attempt to make this approach viable, the pilin gene of *D. nodosus* was cloned and expressed in *E. coli* (25). The recombinant pilin protein was found associated with the inner membrane. When a vaccine consisting of sonicated *E. coli* cells expressing the recombinant pilin was tested in a challenge experiment, the recombinant *E. coli* cells generated an antibody titer similar to that seen for purified, native pili (25). However, the agglutination titer induced by the recombinant *E. coli* cell vaccine was significantly lower than that seen for the intact pili (690 vs. 47,800) and below the titer which correlated with protection (5,000-10,000) (25,26).

Following active challenge with *D. nodosus*, the recombinant pilin vaccine failed to show any significant protective activity, in contrast to that seen for the intact pili. Emery and co-workers had demonstrated that denaturation of intact pili abolished the ability of pilin to induce protection in animals (27). In addition, pili dissociated with either detergent (octyl-β-D-glucoside) or low pH (2.2) reduced the effectiveness of the protein to elicit protection against formation of severe lesions following challenge (28). This was despite the fact that the antibody titers were not significantly different between the groups. The authors stated that "there may be one or more epitopes associated with quaternary structure which are disturbed by the treatments". They further stated:

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"The failure of the *E. coli* expressed product
as a vaccine may result from its physical
occlusion in the host cell membranes, although
preliminary experiments indicated that this was
not the major cause of its ineffectiveness
(unpublished data). An alternative explanation
for the failure of the *E. coli* expressed
product as a vaccine, is that the monomeric
prepilin units which are expressed are unable
to associate into a native conformation for the
appropriate presentation of important epitopes,
possibly because of the presence of the leader
sequence." (28).

Elleman and co-workers confirmed the importance of the
presence of conformational epitopes on the recombinant
pilin and that they needed to increase the immune
response by a better presentation of the antigen. They
proposed two approaches: purification of the protein
from the *E. coli* membranes, or expression of protein in
P. aeruginosa so that it could assemble into
filamentous pili on the surface of the cell. The
latter approach was preferred because "[t]his should
greatly improve the immunogenic properties and simplify
the purification of the protein" (25).

Furthermore, Elleman and co-workers also
viewed the use of pilin (the subunit protein of pili)
as an inferior vaccine candidate to the mature fimbriae
(intact pili) for *D. nodosus*:

"Mature fimbriae appear to provoke a more intense
and appropriate (*i.e.*, K-agglutinating)
immunological reaction than the equivalent dose of
fimbrial subunit protein. A serological K-
agglutination titre of about 5,000 is generally
regarded as the minimum response commensurate with
adequate protective immunity against infection

with a given strain of *B. nodosus*. This level of
response (and up to an order of magnitude higher)
is readily achieved upon vaccination with mature
fimbriae, but not the isolated subunit protein,
which elicits only poor levels of serum K-
agglutinating antibodies" (23).
Additional data suggesting that the fimbrial (pilus)
subunit protein is not a viable vaccine candidate was
recently reported by Alves et al. (29). When mice were
immunized with a polynucleotide vaccine encoding the *E.
coli* CFA/I fimbrial adhesin protein (e.g., pilin), the
antibodies induced were distinct from those induced by
native, intact CFA/I fimbriae. Moreover, these
antibodies against the recombinant protein did not
exhibit any agglutination activities in contrast to
antisera against the native protein.

Despite all the work described above, there
is yet to be developed an effective pilus-based
gonococcal or meningococcal vaccine. Meningococcal
vaccines are limited to those possessing serotype A, C,
Y, W135 capsules.

Accordingly, there is a need to identify
components for inclusion in vaccines to protect against
disease caused by *N. gonorrhoeae* or all serotypes of *N.
meningitidis*.

Summary of the Invention

Thus, it is an object of this invention to
identify suitable antigenic structures derived from *N.
gonorrhoeae* and *N. meningitidis*, respectively, which
may constitute viable vaccine candidates against those
bacteria. These candidates must induce antibodies
which recognize and bind to diverse isolates of the
respective pathogenic neisserial organism.

These and other objects of the invention as discussed below are achieved by the cloning and expression of the recombinant pilin protein (rpilin) of each of *N. gonorrhoeae* and *N. meningitidis*.

5   This invention also relates to the construction of a plasmid which expresses a recombinant meningococcal chimeric class I pilin protein in which the amino-terminal region of the class I meningococcal pilin protein is replaced by the corresponding amino-

10   terminal region of the gonococcal pilin protein. This plasmid expresses significantly higher amounts of the meningococcal chimeric class I rpilin protein than the class I meningococcal rpilin protein expressed from a full-length meningococcal *pil*E gene.

15   In order to obtain expression of the meningococcal chimeric class I rpilin protein, the chimeric DNA sequence is first inserted into a suitable plasmid vector. A suitable host cell is then transformed or transfected with the plasmid. In an

20   embodiment of this invention, the host cell is an *Escherichia coli* strain. The host cell is then cultured under conditions which permit the expression of said chimeric class I rpilin protein by the host cell.

25   This invention further relates to the construction of a plasmid which expresses a recombinant meningococcal chimeric class II pilin protein in which the carboxy-terminal region of the class II meningococcal pilin protein is replaced by the

30   corresponding carboxy-terminal region of the gonococcal pilin protein.

In order to obtain expression of the meningococcal chimeric class II rpilin protein, the chimeric DNA sequence is first inserted into a suitable

35   plasmid vector. A suitable host cell is then

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transformed or transfected with the plasmid. In an embodiment of this invention, the host cell is an *Escherichia coli* strain. The host cell is then cultured under conditions which permit the expression of said chimeric class II rpilin protein by the host cell.

In another embodiment of this invention, the isolated and purified rpilin protein (either the gonococcal, the meningococcal or chimerics) is used to prepare a vaccine composition which elicits a protective immune response in a mammalian host. The vaccine composition may further comprise an adjuvant, diluent or carrier. Examples of such adjuvants include aluminum hydroxide, aluminum phosphate, MPL™, Stimulon™ QS-21, IL-12 and cholera toxin. The vaccine composition is administered to a mammalian host in an immunogenic amount sufficient to protect the host against disease caused by *N. gonorrhoeae* or *N. meningitidis*.

Brief Description of the Figures

Figure 1 depicts transmission electron micrographs of piliated cells from *N. gonorrhoeae* (strain I756 recA-) incubated with guinea pig antisera directed against gonococcal rpilin (from strain Pgh3-1) (1:50 dilution for 15 minutes), followed by donkey anti-guinea pig IgG conjugated to 12 nm colloidal gold (1:5 dilution for 30 minutes and stained with NanoVan. Figure 1A depicts anti-rpilin guinea pig immune sera (week 6); Figure 1B depicts normal guinea pig sera (week 0); Figure 2C depicts no primary antibody.

Figure 2 depicts the effect of guinea pig antisera directed against gonococcal rpilin (from strain Pgh3-1) on the attachment of piliated *N.*

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gonorrhoeae cells (strain I756 recA-) to human cervical
cells (ME180 cell line). Figure 2A depicts the
inhibition of attachment by guinea pig antisera
directed against rpilin (week 6); Figure 2B depicts the
inability of normal guinea pig antisera to prevent
attachment of piliated gonococcal cells to cervical
cells (week 0). Representative sized clumps of
bacteria bound to cervical cells are circled in each
panel. Each panel shows four different views of the
same experimental condition. The guinea pig antisera
was diluted 1:10,000 for each panel.

   Figure 3 depicts transmission electron
micrographs of piliated cells from *N. meningitidis*
(strain H355) incubated with guinea pig antisera
directed against meningococcal chimeric class I rpilin
(from strain H44/76) (1:60 dilution for 30 minutes),
followed by donkey anti-guinea pig IgG conjugated to 12
nm colloidal gold (1:5 dilution for 30 minutes) and
stained with NanoVan. Figure 3A depicts anti-rpilin
guinea pig immune sera (week 6); Figure 3B depicts
normal guinea pig sera (week 0); Figure 3C depicts no
primary antibody. Cells were fixed before being
incubated with antisera.

   <u>Detailed Description of the Invention</u>

   This invention relates to vaccine
compositions comprising a recombinant pilin protein of
N. gonorrhoeae or *N. meningitidis*. Notwithstanding the
teachings of the art discussed above, it was decided to
investigate the use of such recombinant pilin proteins
expressed in *E. coli*. Surprisingly, these recombinant
pilin proteins demonstrated characteristics of vaccine
candidates.

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        The first report describing the cloning of
the gonococcal *pil*E gene in *E. coli* was in 1982 (30).
Since then, molecular characterization of *pil*E has been
performed by numerous laboratories investigating the
genetic factors controlling the expression of the pilin
protein, transport of the pilin protein, variation in
the pilin sequence and the host adherence properties of
pili. However, none of the reports described the
purification of recombinant pilin protein nor the
immune response of the recombinantly expressed pilin
protein.

        Cloning and expression of the *pil*E gene
encoding the gonococcal recombinant pilin protein are
described in Example 2 below. Expression was achieved
by transforming an *E. coli* strain designated TOP10F'
with a plasmid containing the *pil*E gene. Successful
cloning and expression was followed by the sequencing
of the *pil*E gene to confirm identity with the native
sequence. To assist in cloning, a *Nco*I site was
introduced, which required modifying one base. As a
result, the second amino acid in the seven amino acid
long signal peptide was changed from asparagine to
aspartic acid.

        The plasmid containing the *pil*E gene in
Example 2 (designated pPX2000) contains an ampicillin
resistance (Amp^R) marker. As described in Example 3,
another plasmid was constructed to contain a kanamycin
resistance (Kan^R) marker instead of Amp^R. This
plasmid, designated pPX2002, after transforming *E. coli*
strain TOP10F', expressed the gonococcal rpilin at a
level similar to that obtained from pPX2000, which
contains an Amp^R marker.

        As described in Example 4, a similar
procedure was used to construct a plasmid, designated
pPX2003, containing the class I *pil*E gene of *N.*

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meningitidis. A *Nco*I site (CC ATG G) was introduced spanning the beginning of the gene encoding the signal peptide. This changed the second amino acid residue of the signal peptide from asparagine to aspartic acid (the first residue remained methionine). An Amp^R marker was also included. This construct, after transforming *E. coli* strain TOP10F', expressed class I rpilin of *N. meningitidis*. However, the expression level was significantly lower than that for the gonococcal rpilin obtained from either pPX2000 or pPX2002. Without being bound by theory, this lower expression level may be due to a number of inverted repeats which are present in the recombinant class I *pil*E.

In order to increase the expression of the meningococcal pilin, as described in Example 5, a chimeric plasmid was constructed. The DNA in pPX2003 encoding the first 60 amino acids of the meningococcal class I rpilin is replaced with the equivalent region from the gonococcal DNA in pPX2002. The resulting Amp^R plasmid, designated pPX2004, has the nucleotide sequence set forth in SEQ ID NO:1. The plasmid pPX2004 was used to transform an *E. coli* strain K12 designated TOP10F'. Following induction, there was a significant increase in expression of the chimeric rpilin compared to the amount of meningococcal rpilin expressed from pPX2003. The level of expression of the chimieric construct was comparable to the amount of gonococcal rpilin expressed from pPX2002. The chimeric class I rpilin was 167 amino acids in length (including the signal) (SEQ ID NO:2), which is in accordance with the predicted size.

Samples of the *E. coli* strain K12 designated TOP10F' harboring the recombinant plasmid pPX2004 were deposited on January 27, 1998 by the Applicants with

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the American Type Culture Collection, 10801 University
Boulevard, Manassas, Virginia 20110-2209, U.S.A., and
have been assigned ATCC accession number ATCC 98637.

As described in Example 6, a chimeric plasmid
5   was constructed wherein the 3' end of the class II *pilE*
gene of *N. meningitidis* was replaced with the
corresponding region from *N. gonorrhoeae*.
Specifically, the DNA in pPX8001 encoding the disulfide
loop (the last 22 amino acids of the meningococcal
10   class II pilin of *N. meningitidis* strain FAM18) is
replaced with a similiar (but larger) region plus
additional portions of the carboxy-terminal region
totalling 44 amino acids from the gonococcal (*pilE*) DNA
from *N. gonorrhoeae* strain Pgh3-1 in pPX2000. The
15   resulting Amp^R plasmid, designated pPX8017, has the
nucleotide sequence set forth in SEQ ID NO:3, in which
nucleotides 1-378 are from *N. meningitidis* class II and
nucleotides 379-510 are from *N. gonorrhoeae*. The
plasmid pPX8017 was used to transform the *E. coli*
20   strain K12 designated TOP10F'. Following induction, a
chimeric class II rpilin was expressed which was 170
amino acids in length (including the seven amino acid
long signal) (SEQ ID NO:4), in which amino acids 1-126
are from *N. meningitidis* class II and amino acids 127-
25   170 are from *N. gonorrhoeae*. This chimeric class II
rpilin was in accordance with the predicted size. A
*Nco*I site was introduced for cloning considerations,
which changed the second amino acid in the signal
sequence from lysine to glutamic acid. This change was
30   not expected to have any effect on antigenicity or
immunogenicity.

Samples of the *E. coli* strain K12 designated
TOP10F' harboring the recombinant plasmid pPX8017 were
deposited on April 15, 1999 by the Applicants with the
35   American Type Culture Collection, 10801 University

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Boulevard, Manassas, Virginia 20110-2209, U.S.A., and
have been assigned ATCC accession number ATCC 207199.

A variety of host cell-vector systems are
suitable for use to express the gonococcal,
meningococcal and chimeric rpilins used in the vaccines
of this invention in addition to those detailed in
Examples 2-6. The vector system is compatible with the
host cell used. Suitable host cells include bacteria
transformed with plasmid DNA, cosmid DNA or
bacteriophage DNA; viruses such as vaccinia virus and
adenovirus; yeast such as Pichia cells; insect cells
such as Sf9 or Sf21 cells; or mammalian cell lines such
as Chinese hamster ovary cells; as well as other
conventional organisms.

A variety of conventional transcriptional and
translational elements can be used for the host cell-
vector system. The *pil*E DNA is inserted into an
expression system and the promoter and other control
elements are ligated into specific sites within the
vector, so that when the plasmid vector is inserted
into a host cell, the *pil*E DNA can be expressed by the
host cell.

The plasmid is introduced into the host cell
by transformation, transduction, transfection or
infection, depending on the host cell-vector system
used. The host cell is then cultured under conditions
which permit expression of the rpilin protein by the
host cell.

This invention further relates to an isolated
and purified DNA sequence comprising a DNA sequence
encoding the meningococcal chimeric class I rpilin
protein whose amino-terminal region is from the
gonococcal *pil*E gene and whose central and carboxy-
terminal regions are from the meningococcal *pil*E gene
(SEQ ID NO:1). Nucleotides 1-501 in SEQ ID NO:1 encode

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the meningococcal chimeric class I rpilin protein prior to processing; nucleotides 22-501 encode the meningococcal chimeric class I rpilin protein after processing to a mature protein. The invention additionally relates to the meningococcal chimeric class I rpilin protein having the amino acid sequence of amino acids 1-167 of SEQ ID NO:2 prior to processing or having the amino acid sequence of amino acids 8-167 of SEQ ID NO:2 after processing to a mature protein. Approximately 10% of the total protein produced by the gonococcal rpilin or the meningococcal chimeric class I rpilin constructs lacks the signal sequence, which has been removed by processing.

This invention further relates to an isolated and purified DNA sequence comprising a DNA sequence encoding the meningococcal chimeric class II rpilin protein whose carboxy-terminal region is from the gonococcal *pilE* gene and whose central and amino-terminal regions are from the meningococcal *pilE* gene (SEQ ID NO:3). Nucleotides 1-510 in SEQ ID NO:3 encode the meningococcal chimeric class II rpilin protein prior to processing; nucleotides 22-510 encode the meningococcal chimeric class II rpilin protein after processing to a mature protein. The invention additionally relates to the meningococcal chimeric class II rpilin protein having the amino acid sequence of amino acids 1-170 of SEQ ID NO:4 prior to processing or having the amino acid sequence of amino acids 8-170 of SEQ ID NO:4 after processing to a mature protein.

In addition to the chimeric DNA sequences contained in pPX2004 and pPX8017 which encode the meningococcal chimeric class I rpilin protein and the meningococcal chimeric class II rpilin protein, respectively, the present invention further comprises DNA sequences which, by virtue of the redundancy of the

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genetic code, are biologically equivalent to the sequences which encode for the chimeric rpilin proteins, that is, these other DNA sequences are characterized by nucleotide sequences which differ from those set forth herein, but which encode a protein having the same amino acid sequence as that encoded by the DNA sequence in SEQ ID NO:1 or SEQ ID NO:3.

In particular, the invention contemplates those DNA sequences which are sufficiently duplicative of the sequence of SEQ ID NO:1 or SEQ ID NO:3 so as to permit hybridization therewith under standard high stringency Southern hybridization conditions, such as those described in Sambrook et al. (31).

This invention also comprises DNA sequences which encode amino acid sequences which differ from those of the meningococcal chimeric class I or class II rpilin proteins, but which are biologically equivalent to those described for one of these proteins (SEQ ID NO:2 or SEQ ID NO:4). Such amino acid sequences may be said to be biologically equivalent to those of the chimeric rpilin protein if their sequences differ only by minor deletions from, insertions into or substitutions to the rpilin sequence, such that the tertiary configurations of the sequences are essentially unchanged from those of the rpilin protein.

For example, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, as well as changes based on similarities of residues in their hydropathic index,

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can also be expected to produce a biologically
equivalent product. Nucleotide changes which result in
alteration of the N-terminal or C-terminal portions of
the protein molecule would also not be expected to
5   alter the activity of the protein.
          Furthermore, changes in known variable
regions are biologically equivalent where the tertiary
configurations of the conserved regions are essentially
unchanged from those of the rpilin protein. An
10   alternative definition of a biologically equivalent
sequence is one that is still capable of generating a
cross-reactive immune response. In particular, the
meningococcal chimeric class I and II recombinant
pilins may be modified by lengthening or shortening the
15   corresponding insertion from the gonococcal pilin, as
long as the modified chimeric recombinant pilin is
still capable of generating a cross-reactive immune
response.
          Each of the proposed modifications is well
20   within the routine skill in the art, as is determi-
nation of retention of structural and biological
activity of the encoded products. Therefore, where the
terms "meningococcal chimeric class I rpilin protein"
or "meningococcal chimeric class II rpilin protein" are
25   used in either the specification or the claims, it will
be understood to encompass all such modifications and
variations which result in the production of a
biologically equivalent protein.
          As described in Example 7, the gonococcal
30   rpilin protein is associated with cellular membranes of
the E. coli used to express it. A variety of
detergents are able to selectively solubilize the
rpilin protein from E. coli, including Empigen™ BB,
Triton™ X-100, reduced Triton™ X-100, octyl-β-D-
35   glucopyranoside (OG), Zwittergent™ 3-10 or 3-14.

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Following centrifugation, dialysis and fractionation on a column, the purified rpilin is obtained.

As described in Example 8, the chimeric class I rpilin was isolated and purified by disruption of *E. coli* cells, clarification by centrifugation, filtration, and fractionation on two columns.

As described in Example 9, the meningococcal chimeric class II rpilin was isolated and purified by disruption of *E. coli* cells, clarification by centrifugation, dialysis, and fractionation on two columns.

The purified gonococcal rpilin was subjected to repeated N-terminal sequencing as described in Example 10. Sequencing of the 20-40 amino-terminal residues gave results which agreed with the amino acid sequence deduced from the DNA sequence. The molecular weight of the rpilin (with signal) was determined to be 18,006 daltons by mass spectrometry, which compares well to the predicted mass of 17,981 daltons based on the amino acid content. In contrast, an apparent molecular weight of 68,899 daltons was obtained when the rpilin was subjected to size exclusion column chromatography using detergent. This suggested that the rpilin aggregated. Dialysis of the rpilin against PBS in an effort to remove detergent resulted in material having an apparent molecular weight of 452,349 daltons, as measured by gel filtration. This suggested that it had undergone further aggregation.

As detailed in Example 11, immune sera are obtained by immunizing guinea pigs or mice with the purified gonococcal rpilin. As set forth in Example 12, Western blot analysis showed that antisera against rpilin bound to whole cell lysates from piliated gonococcal cells, while there was no binding seen in non-piliated cell lysates. In contrast, antisera to

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the pilin oligomer bound to both piliated and
nonpiliated cell lysates.

As detailed in Example 13, when analyzed by
ELISA, this pooled antisera against gonococcal rpilin
had high endpoint titers for binding to purified
gonococcal rpilin protein. Example 13 also details the
effects of various adjuvants. When the rpilin was
adjuvanted with either MPL™ alone, MPL™ plus aluminum
phosphate, or Stimulon™ QS-21, good humoral immune
responses in mice were obtained.

As set forth in Example 14, whole cell ELISA
showed that antisera against rpilin bound to piliated
cells, but not to isogenic non-piliated cells of a
particular gonococcal strain.

As described in Example 15, mice were
immunized intranasally with gonococcal rpilin with or
without native cholera toxin. There was a significant
immune response detected in the antigen ELISA from
pooled sera generated after the mice were immunized
with rpilin in the absence of adjuvant; this response
was enhanced by the addition of native cholera toxin.
The pooled sera had a low ELISA titer for binding to
intact, piliated gonococcal cells; this binding was
greatly enhanced when the mice were also immunized with
native cholera toxin.

As described in Example 16, immunoelectron
microscopy demonstrated that antibodies against rpilin
were bound along the length of the pili filaments on
the surface of gonococci. This suggested that the
antibodies bound to epitopes which would be present on
the surface of the bacteria *in vivo*.

Example 17 demonstrates the higher titers
obtained for rpilin antisera binding to heterologous
piliated bacterial isolates as compared to that
obtained for antisera to recombinant pilin oligomer.

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The rpilin is converted to rpilin oligomer by dialysis of the rpilin against pH 12 phosphate buffer.

Pili mediate the initial binding of *N. gonorrhoeae* to human mucosal cells. Therefore, if an antigen is able to elicit antibodies which inhibit the attachment of these bacteria to those cells, this would provide evidence that such an antigen is a vaccine candidate.

As discussed in Example 18, guinea pig antisera to rpilin significantly inhibited the binding of gonococci expressing heterologous pili to human cervical epithelial cells. Piliation of gonococci correlates with the infectivity of this bacterium (2,3,32).

These data indicate that the recombinant pilin was able to generate antibodies which bind to diverse pili on intact gonococcal cells and that the antisera exhibits a functional activity (inhibition of bacterial adherence) which would protect immunized human beings against gonococcal colonization and infection (32,33). It has been previously reported that immunization with *E. coli* cells which expressed recombinant pilin from *D. nodosus* were immunogenic (23,25,28), but not protective against challenge. Because of these results, these researchers turned away from the use of recombinant subunit pilin in favor of the assembled pilus. Yet, the data described herein suggest that, following purification, the recombinantly expressed pilin protein induces an immune response which should correlate with protection of humans from gonococcal colonization. Thus, these data support the view that rpilin is a viable vaccine candidate against *N. gonorrhoeae*.

As described in Example 19, the meningococcal chimeric class I rpilin protein was subjected to N-

terminal sequencing. Sequencing of the 35 amino-
terminal residues gave results which agreed with the
amino acid sequence deduced from the DNA sequence. The
molecular weight of the chimeric rpilin (with signal)
5   was determined to be 17,659 daltons by mass
spectrometry, which compares well to the predicted mass
of 17,676 daltons based on the amino acid content. In
contrast, an apparent molecular weight of 69,480
daltons was obtained when the meningococcal chimeric
10   class I rpilin protein was subjected to size exclusion
column chromatography using detergent. As with the
gonococcal rpilin, this suggested that the
meningococcal chimeric class I rpilin protein
aggregated.

15   As detailed in Example 20, when analyzed by
ELISA, pooled antisera against the meningococcal
chimeric class I rpilin protein had high endpoint
titers to both meningococcal class I rpilin protein and
to piliated meningococcal cells. As also detailed in
20   Example 20, adjuvants, in particular Stimulon™ QS-21,
generated significant responses for the binding of
antisera against the meningococcal chimeric class I
rpilin protein to both meningococcal class I rpilin
protein and to piliated meningococcal cells.

25   As described in Example 21, immunoelectron
microscopy demonstrated that antibodies against the
meningococcal chimeric class I rpilin protein were
bound along the length of the pili of meningococci.
This suggested that the antibodies bound to epitopes
30   which would be present on the surface of the bacteria
in vivo.

In Example 4, it was shown that antisera
directed against gonococcal rpilin recognized and bound
to piliated meningococcal cells. In Example 22, it was
35   shown that antisera raised against meningococcal

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chimeric class I rpilin protein bound to piliated
gonococcal cells.

As described in Example 23, passive
immunization of infant rats with guinea pig antisera
against meningococcal chimeric class I rpilin protein
can help prevent meningococcal bacteremia *in vivo*.
There was a significant decrease in the level of
colonization in animals who received this immune sera.
Furthermore, the immune response generated using
recombinantly expressed pilin can protect, *in vivo*,
against meningococci which express a heterologous pilin
protein.

As described in Example 24, mice were
immunized intranasally with meningococcal chimeric
class I rpilin with or without cholera toxin, where the
cholera toxin is in a mutant form wherein the glutamic
acid at amino acid position 29 is replaced by a
histidine (CT-CRM, E29H). There was a significant
immune response detected in the antigen ELISA from
pooled sera generated after the mice were immunized
with rpilin in the absence of adjuvant; this response
was enhanced by the addition of mutant CT-CRM, E29H
cholera toxin.

As described in Example 25, the inhibition of
colonization of mouse nasopharynx by a class I strain
of *N. meningitidis* was demonstrated in mice immunized
subcutaneously with meningococcal chimeric class I
rpilin adjuvanted with MPL™.

As described in Example 26, Western blot
analysis showed that antisera obtained from guinea pigs
immunized with meningococcal chimeric class II rpilin
bound to whole cell lysates from piliated meningococcal
cells which expressed either class I or class II pilin.

As described in Example 27, antisera elicited
against partially purified meningococcal chimeric class

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II rpilin bound to meningococcal cells from the
homologous bacterial strain.

Taken together, these data support the view
that rpilin, in particular the meningococcal chimeric
class I and class II rpilin proteins, are viable
vaccine candidates against *N. meningitidis*.

The gonococcal rpilin protein is useful in
the preparation of vaccines to confer protection to
mammals against disease caused by *N. gonorrhoeae*. The
meningococcal rpilin protein, the meningococcal
chimeric class I rpilin protein and the meningococcal
chimeric class II rpilin protein are useful in the
preparation of vaccines to confer protection to mammals
against disease caused by *N. meningitidis*.

In addition, cross-protection against a
different *Neisseria* species is afforded by immunizing
with a vaccine containing the gonococcal rpilin protein
to confer protection to mammals against disease caused
by *N. meningitidis* or by immunizing with a vaccine
containing the meningococcal rpilin protein, the
meningococcal chimeric class I rpilin protein or the
meningococcal chimeric class II rpilin protein to
confer protection to mammals against disease caused by
N. gonorrhoeae.

These vaccine compositions comprise an
isolated and purified rpilin protein, wherein the
vaccine composition elicits a protective immune
response in a mammalian host.

Vaccines containing a rpilin protein may be
mixed with immunologically acceptable diluents or
carriers in a conventional manner to prepare injectable
liquid solutions or suspensions. The level of
antibodies elicited by the vaccine may be improved by
using certain adjuvants such as Stimulon™ QS-21 (Aquila
Biopharmaceuticals, Inc., Framingham, MA), MPL™ (3-O-

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deacylated monophosphoryl lipid A; RIBI ImmunoChem
Research, Inc., Hamilton, MT), aluminum phosphate,
aluminum hydroxide, IL-12 (Genetics Institute,
Cambridge, MA) and cholera toxin (either in a wild-type
or mutant form, for example wherein the glutamic acid
at amino acid position 29 is replaced by another amino
acid, preferably a histidine, in accordance with U.S.
Provisional Patent Application Number 60/102,430).

The vaccines of this invention are
administered by injection in a conventional manner,
such as subcutaneous, intraperitoneal or intramuscular
injection into humans, as well as by oral, mucosal,
intranasal or vaginal administration, to induce an
active immune response for protection against disease
caused by *N. gonorrhoeae* or *N. meningitidis*. The
dosage to be administered is determined by means known
to those skilled in the art. Protection may be
conferred by a single dose of vaccine, or may require
the administration of several booster doses.

In order that this invention may be better
understood, the following examples are set forth. The
examples are for the purpose of illustration only and
are not to be construed as limiting the scope of the
invention.

Examples

Standard molecular biology techniques are
utilized according to the protocols described in
Sambrook et al. (31).

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Example 1
Bacteria and Cell Cultures

Bacteria and Culture Conditions

5   The gonococcal isolates were obtained from
Tampa, FL; Ottawa, Canada; Washington, D.C.; Seattle,
WA; Rochester, NY; Chapel Hill, NC; and Evanston, IL.
The meningococcal isolates were obtained from Chapel
Hill, NC; and Bilthoven, Netherlands.

10   The bacteria were stored in lyophilized form
or frozen at -70°C until required. When grown on solid
media, the agar plates were incubated in an incubator
at 37°C containing a humidified atmosphere and 5% (v/v)
CO_2. *N. gonorrhoeae* and *N. meningitidis* were grown on

15   GC medium base (Difco Laboratories, Detroit, MI)
without hemoglobin, but supplemented with dextrose (400
mg/L), glutamine (10 mg/L), cocarboxylase (20 μg/L) and
ferric nitrate (500 μg/L). Liquid suspension cultures
of *N. meningitidis* were grown in the same media which

20   lacked agar in a shaking incubator (70 RPM) at 37°C.
In experiments involving culturing of meningococci from
mouse nasal tissue homogenates, the bacteria were grown
on GC media described above with the following mixture
of antibiotics (Difco): colistin sulfate (75 μg/mL),

25   nystatin (125 μg/mL), vancomycin (30 μg/mL) and
trimethoprim lactate (50 μg/mL). Piliated gonococci
were identified by colony morphology and individual
colonies passaged daily in order to maintain the
phenotype. The piliation state of meningococcal cells

30   was assessed by transmission electron microscopic
examination of samples stained with NanoVan stain
(Nanoprobes, Stony Brook, NY) at pH 8 for 30 seconds.
E. coli were grown on SOB agar which consists of 20 g/L
Bacto tryptone (Difco), 5 g/L yeast extract (Difco),

35   0.6 g/L NaCl, 0.2 g/L KCl and 1% (w/v) agar (pH 7.5) or

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in SOB broth to which the agar is not added. For some
experiments, the Bacto tryptone was replaced with an
equivalent amount of HySoy™ (Sheffield Products,
Norwich, NY).

5

Epithelial cell cultures

        ME-180 cell line (ATCC, Beltsville, MD) is an
epidermoid carcinoma which was originally derived from
10   a cervical carcinoma. The cells were grown in RPMI
1640 (Gibco BRL, Gaithersburg, MD) supplemented with
10% (v/v) fetal calf serum (Sigma, St. Louis, MO),
penicillin G (1000 units/mL) (Gibco BRL), L-
streptomycin (1 mg/mL) (Gibco BRL) and 2 mM L-glutamine
15   in a humidified atmosphere of 5% (v/v) CO_2 at 37°C.
The cells were split every three to four days.

Example 2
Cloning and Expression of Gonococcal pilE in E. coli

20

        A frozen sample of piliated N. gonorrhoeae
strain Pgh3-1 was used as the source of the pilE DNA in
a PCR reaction. The pilE gene was amplified using the
following primers which recognized the 3' and 5' ends of
25   the complete pilin protein (including the leader
sequence): 5' CCC|CGC GCC ATG GAT ACC CTT CAA AAA GGC
3' (PILEFWD) (SEQ ID NO:5) and 5' GGG CCT GGA TCC GTG
GGA AAT CAC TTA CCG 3' (PILEREV) (SEQ ID NO:6). The
resulting PCR product contained a NcoI site at the
30   beginning of the pilE coding region and a BamHI site at
the end. The NcoI site was introduced into the gene
because of cloning considerations. This resulted in a
change of the second amino acid in the signal sequence
from asparagine (AAT) to aspartic acid (GAT). Because
35   amino acid 2 is part of the signal peptide which is

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cleaved during normal processing of the mature protein,
this change was not expected to have any effect on
antigenicity or immunogenicity. The PCR product was
cloned into a pCR™II TA cloning vector (Invitrogen,
5   Carlsbad, CA), ligated, and transformed into *E. coli*
TOP10F' (Invitrogen). Colonies were selected on 100
µg/mL ampicillin containing plates or on 50 µg/mL
kanamycin containing plates. The plasmid DNA was
isolated from overnight cultures of these transformants
10   and analyzed by restriction digests using the enzymes
*Eco*RI and *Not*I.

Four clones containing an insert of the
correct size were submitted for DNA sequence analysis
in order to verify the presence of *pil*E PCR DNA
15   fragment. Clone #17, designated pPX1999, was used as
the source of the *pil*E gene. Plasmid DNAs from pPX1999
and pTrcHisA (Invitrogen) were digested with *Nco*I and
*Bam*HI restriction enzymes, the DNA fragments gel
isolated, ligated and transformed into *E. coli* TOP10F'.
20   Ampicillin resistant colonies were selected, the
plasmid DNA of the new transformant isolated, and a DNA
restriction analysis done using *Bam*HI and *Nco*I
restriction enzymes. Two clones with the correct
restriction pattern were submitted for DNA sequence
25   analysis. Both clones had the correct DNA sequence and
were designated pPX2000.

To test for expression of the recombinant
pilin, cultures containing these clones were grown in
either shake flasks or a fermentor in SOB plus 100
30   µg/mL ampicillin and 12 µg/mL tetracycline.
When shake flasks were used, *E. coli* were grown in 1L
of media until an A_{600} = 0.9-1.0 was obtained. The
expression of the recombinant pilin was induced by the
addition of isopropyl-β-D-thiogalactopyranoside (IPTG)
35   to a final concentration of 1 mM and allowing growth to

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continue for 1-4 hours, at which point the cells were
collected by centrifugation (13,689 x g for 20 minutes
at 4°C) and stored at -20°C. For the fermentor, an
overnight culture from a plate was used to inoculate a
flask containing 500 mL media which was again grown
overnight. This liquid culture was then used to
inoculate a Biostat B Fermentor (Braun Biotech,
Allentown, PA) containing 8.9 L of media. Enhanced
growth of the bacteria in the fermentor was obtained
when HySoy™-containing media was supplemented with
dextrose at a final concentration of 1% (w/v). When
the culture reached A_{600} = 1.0, IPTG was added to a
final concentration of 1 mM and the cells were allowed
to grow for another 1-4 hours before being harvested by
centrifugation (13,689 x g for 20 minutes at 4°C). The
media was discarded and the cell pellet stored at
-20°C. Upon induction with IPTG, expression of rpilin
protein increased significantly and reached maximal
levels at three to four hours post induction.

        Samples of the induced cultures were analyzed
by polyacrylamide gel electrophoresis in the presence
of sodium dodecyl sulfate (SDS-PAGE). The recombinant
pilin was visualized using Coomassie blue staining and
its identity confirmed by Western blot with a
monoclonal antibody specific for gonococcal pili (clone
#A33020023, Biospacific, Emeryville, CA).

                        Example 3
      Construction of Gonococcal Recombinant *pilE* Plasmid
              Containing Kanamycin Resistance Marker

        A plasmid was constructed where the Amp^R
marker was replaced with a Kan^R marker. Except as
noted below, the procedures of Example 2 were used. A
PCR reaction was performed on pTrcHisA plasmid DNA

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using TrcFXba primer, 5' GGC TCT AGA CTG TCA GAC CAA
GTT TAC TC 3' (SEQ ID NO:7), and TrcRXba primer, 5' GGC
TCT AGA TTG AAG CAT TTA TCA GGG 3' (SEQ ID NO:8). The
underlined sequences code for an XbaI restriction site.
The approximate 3.5 kb PCR product contained the
pTrcHisA DNA minus the ampicillin coding region.
Another PCR reaction was performed on pACYC177 plasmid
DNA (New England Biolabs, Beverly, MA) using KanFXba
primer, 5' GGC TCT AGA TAA ACA GTA ATA CAA GGG G 3'
(SEQ ID NO:9), and KanRXba primer, 5' GGC TCT AGA TTA
GAA AAA CTC ATC GAG C 3' (SEQ ID NO:10). Again, the
underlined sequences code for an XbaI restriction site.
The approximate 860 bp PCR DNA product contains the
kanamycin resistance gene. Both PCR products were
purified from an agarose gel, the DNAs were digested
with the XbaI restriction enzyme, extracted and ligated
together. An aliquot of the ligation reaction was
transformed into E. coli TOP10F' and an aliquot of the
transformation mix plated on SOB plates containing 30
μg/mL of kanamycin.

A total of 48 Kan^R colonies were streaked in
duplicate onto SOB plates containing either ampicillin
or kanamycin. As anticipated, all Kan^R colonies were
ampicillin sensitive. Since the cloning design was
symmetrical, both orientations of the kanamycin insert
were isolated. The kanamycin insert in the same
clockwise orientation as the original ampicillin gene
was selected for future studies and called pZ564. The
DNA region containing the lacIq gene, trc promoter and
the multiple cloning site in pZ564 was then replaced
with the similar region from the pPX2000 plasmid (which
also contained pilE) in the following manner: Both
pZ564 and pPX2000 were digested with SphI and XmnI
restriction enzymes. The approximate 2.2 kb DNA
fragment from pPX2000 and the approximate 2.6 kb DNA

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fragment from pZ564 were gel purified, ligated together
and transformed into *E. coli* TOP10F'. The resulting
correct plasmid was called pPX2002.

5   A similar time course of induction and level
of recombinant pilin protein expression was seen when
the selection antibiotic was changed to kanamycin from
ampicillin.

                              Example 4
10   Cloning and Expression of Meningococcal
                        Class I *pil*E in *E. coli*

         Because of the highly homologous nature of
the DNA and amino acid sequences of gonococcal and
15   meningococcal class I pilins (7), the ability of
gonococcal rpilin antisera to bind to pili expressed on
meningococcal cells was assessed using the whole cell
ELISA. This antisera exhibited a titer of 151,100 for
binding to *N. meningitidis* piliated cells from strain
20   H355.
         This binding appeared to be directed against
the pilin protein, since a Western blot of the whole
cell lysate from this strain showed that only a single
band co-migrating with pilin bound the antisera (data
25   not shown). A number of other meningococcal strains
exhibited lower titers in the whole cell ELISA, but the
presence of pili was not confirmed by transmission
electron microscopy. Based on these data, it was
decided to clone and recombinantly express the class I
30   pilin from *N. meningitidis*. Initially, the same
strategy was followed as with the *pil*E from *N.
gonorrhoeae*. Except as noted below, the procedures of
Example 2 were used.
         The class I *pil*E was amplified from the
35   genomic DNA of *N. meningitidis* strain H44/76 using the

following primers: 5' CCC CGC GCC ATG GAC ACC CTT CAA
AAA GGT TTT ACC 3' (NMFPILE) (SEQ ID NO:11), and 5' GGG
CCT GGA TCC GAG TGG CCG TGG AAA ATC ACT TAC CGC 3'
(NMRPILE) (SEQ ID NO:12). As anticipated, a PCR
product of approximately 600 bp DNA was obtained. An
aliquot of the PCR reaction product was digested with
BamHI and NcoI restriction enzymes for insertion into
pTrcHisA. The digested DNAs were electrophoresed on an
agarose gel and the DNA fragments gel purified. The
DNA fragments were then ligated together and
transformed into E. coli TOP10F'. Miniplasmid prep
analysis of ampicillin resistant clones was performed
using BamHI and NcoI restriction enzymes. Clones
expressing the correct restriction digest pattern were
called RZ1142 and the plasmid was called pPX2003.
        Following induction, the presence of a
Coomassie blue stained polypeptide with a molecular
weight of approximately 15,000 daltons was observed
when a whole cell lysate was analyzed by SDS-PAGE.
Analysis of whole cell lysates from four of these
clones by SDS-PAGE and Western blot demonstrated the
presence of a protein of appropriate mobility and
reactivity with polyclonal antisera against the intact
pili from N. gonorrhoeae strain LB2. The purified
class I rpilin was 167 amino acids in length. However,
the level of expression of this recombinant pilin was
significantly lower than that obtained with either
pPX2000 or pPX2002 grown under the same conditions. An
analysis of the DNA sequence in the recombinant class I
pilE showed that there were a number of inverted
repeats which might explain the low level of expression
of this protein in E. coli.

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Example 5
Construction and Expression of a Gonococcal and
Meningococcal Class I Chimeric _pil_E in _E. coli_

5
        In order to increase the expression of the
meningococcal pilin protein, the DNA encoding the first
60 amino acids in pPX2003 (the meningococcal class I
_pil_E construct described in Example 4) was replaced
with the equivalent region from pPX2002 (the gonococcal
10   _pil_E construct described in Example 3, including the
seven amino acid signal peptide) (SEQ ID NO:4). Except
as noted below, the procedures of Example 2 were
followed.

        The conserved 5' terminal region of the
15   meningococcal _pil_E gene was replaced by the same region
from _N. gonorrhoeae_ strain Pgh3-1 in the following
manner. A _Bsm_BI site was introduced into the
meningococcal _pil_E gene as follows: DNA was PCR
amplified from pPX2003 using the following primers: 5'
20   CCG GCG CGT CTC TCA CGG CGA ATG GCC CGG C 3' (CL-1ESPF)
(SEQ ID NO:13) and 5' GGG CCT GGA TCC GAG TGG CCG TGG
AAA ATC ACT TAC CGC 3' (NMRPILE) (SEQ ID NO:14) and Taq
DNA polymerase. The expected PCR DNA product was
cloned directly into pCR2.1 (Invitrogen) and
25   transformed into TOP10F' cells and the resulting
plasmid was designated pZ578. A _Bsm_BI site was then
introduced into the gonococcal _pil_E by the following
method. Using the primers 5' GCA TAA TTC GTG TCG CTC
AAG GCG C 3' (TRCUPFW) (SEQ ID NO:15) and 5' GCC GCG CGT
30   CTC CCG TGA TTC AGG TAA TAC TCG G 3' (PILEESPR) (SEQ ID
NO:16) and Pfu DNA polymerase, the 5' end of the _pil_E
gene from pPX2000 was PCR amplified. The resulting
gonococcal PCR product and pZ578 were then digested
with _Bsm_BI and ligated together. The ligated DNAs were

then PCR amplified using 5' GCA TAA TTC GTG TCG CTC AAG
GCG C 3' (TRCUPFW) (SEQ ID NO:17) and 5' GGG CCT GGA TCC
GAG TGG CCG TGG AAA ATC ACT TAC CGC 3' (NMRPILE) (SEQ
ID NO:18) primers.

5   The DNA PCR product was of the predicted size
(approximately 850 bp) and was digested with NcoI and
BamHI to yield an approximately 600 bp fragment. This
fragment was gel isolated and cloned directly into
NcoI- and BamHI-cut pPX2000 vector, replacing the

10   gonococcal pilE gene. The resulting plasmid, which was
ampicillin resistant, was labeled pPX2004 and used to
transform TOP10F'. Analysis of this transformant
demonstrated the presence of the desired chimeric pilE
DNA. Following induction with IPTG, there was a

15   significant increase in the amount of the meningococcal
chimeric class I rpilin construct expressed compared to
the amount of meningococcal rpilin expressed from
pPX2003. Using the extraction with 1% octyl-β-D-
glucopyranoside (OG) and purification protocol (TMAE

20   Fractogel™ column in 10 mM Tris™, pH 8.5 with 0.1%
(w/v) Zwittergent™ 3-14) described in Example 7 for
the recombinant gonococcal pilin, highly purified
meningococcal chimeric class I rpilin protein was
obtained (yield approximately 5 mg/gram cell wet

25   weight). This material was greater than 90% pure when
analyzed by SDS-PAGE and laser densitometry. SDS-PAGE
demonstrated the presence of a major band of
approximately 15,000 daltons in size. The
meningococcal chimeric class I rpilin protein was also

30   167 amino acids in length, and includes the signal
sequence of seven amino acids as demonstrated by
sequencing of the amino-terminal 36 residues of the
purified protein.

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Example 6
Construction and Expression of a Gonococcal and Meningococcal Class II Chimeric _pil_E in _E. coli_

5   The initial cloning of the meningococcal
class II _pil_E involved isolation of chromosomal DNA
from piliated _N. meningitidis_ strain FAM18 cells and
amplifying the classII _pil_E DNA in a PCR reaction. The
class II _pil_E gene was amplified using the following
10   primers which recognized the 3' and 5' ends of the
complete pilin protein (including the leader sequence):
5' GCG GCC GCC ATG GAA GCA ATC CAA AAA GGT TTC ACC C 3'
(PILE2FWD) (SEQ ID NO:19) and 5' GCG GCC GGA TCC GGT
CAT TGT CCT TAT TTG GTG CGG C 3' (PILE2REV) (SEQ ID
15   NO:23). In a similar strategy as in Example 2, the
resulting PCR product contained an _Nco_I site at the
beginning of the _pil_E coding region and a _Bam_HI site at
the end. The _Nco_I site was introduced into the gene
because of cloning considerations. This resulted in a
20   change of the second amino acid in the signal sequence
from lysine (AAA) to glutamic acid (GAA). As stated
previously, this change was not expected to have any
effect on antigenicity or immunogenicity. The PCR
product was cloned into a pCR2.1 cloning vector
25   (Invitrogen), ligated, and transformed into _E. coli_
TOP10F'. Colonies were selected on 100 μg/mL
ampicillin-containing plates or 50 μg/ml kanamycin
plates. The plasmid DNA was isolated from overnight
cultures of these transformants and analyzed by
30   restriction digests using the enzyme _Eco_RI.
         Clone #8, designated pPX8001, was used as the
source of the _pil_E gene. Plasmid DNAs from pPX8001 and
pTrcHisC (Invitrogen) were each digested with _Nco_I and
_Bam_HI restriction enzymes, and the resulting DNA
35   fragments were gel isolated, ligated and transformed

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into *E. coli* TOP10F'. Following selection of
ampicillin resistant colonies, the plasmid DNA of the
new transformants were isolated, and a DNA restriction
analysis performed using *BamHI* and *NcoI* restriction
enzymes. Two clones with the correct restriction
pattern were submitted for DNA sequence analysis. Both
clones had the correct DNA sequence, designated as
pPX8002.

To test for expression of the recombinant
class II pilin, 10 mL cultures containing these clones,
pPX8002, were grown in 50 mL tubes in SOB containing
100 μg/mL ampicillin and 12 μg/mL tetracycline, to an
A_{600} = 1.0. Expression of the recombinant protein was
induced by adding IPTG to final concentration of 1 mM
and the culture was continued for three hours. When a
whole cell lysate of the induced cells was separated by
SDS-PAGE and stained with Coomassie blue, no new
(induced) band was detected. This suggested that the
FAM18 *pilE* gene product was expressed at levels lower
than those discernable with Coomassie blue. When the
FAM18 *pilE* was cloned into the pET17b plasmid and
transformed into *E. coli* BL21(DE3)pLysS with or without
the *pilE* signal sequence, no significant expression of
recombinant protein was detected. Similar results were
obtained when the class II *pilE* gene from two other
strains of *N. meningitidis* (NmB, 2996) were cloned into
the same pTrcHis plasmid and TOP10F' expression system.

Specifically, the strains NMB and 2996 were
also determined to be expressing the class II pilin,
based on PCR and sequencing data. The *pilE* gene was
amplified from several *N. meningitidis* strains using a
class I set of primers (NMFPILE and NMRPILE) and a
class II set of primers (PILE2FWD and PILE2REV) in
separate reactions with either chromosomal DNA or cells
as the template. PCR products were cloned into

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pTrcHisC and sequenced, or were sequenced directly.
Alignments of sequences were carried out; sequences
similar to those from the H44/76 strain were classified
as class I, while sequences similar to those from the

5   FAM18 strain were classified as class II. Sequences
were not obtained for all the strains amplified. A
preliminary classification was also made based on PCR
data. Class I strains were those which gave a correct
size PCR product with class I primers but not with

10   class II primers, while class II strains were those
which gave a correct size PCR product with class II
primers but not with class I primers.

        Based on the experience with the
meningococcal class I pilin, the region encoding the

15   first 60 amino acids (the conserved amino-terminal
region) of the class II pilE were replaced with the
corresponding region from N. gonorrhoeae strain Pgh3-1.
Expression of the resulting chimeric pilE was
investigated in a number of E. coli expression strains

20   using a variety of promoters. The strains studied
included: PR13 (Rnase deficient), BL21 (protease
deficient), KS474 (deficient in periplasmic protease),
AD494 (Novagen, which allows disulfide bond formation
in cytoplasm) and three strains of TOPP (Stratagene,

25   non-K12 strains useful for hard to express proteins).
In all cases, no recombinant protein was detected in
Coomassie blue- stained SDS-PAGE. An alternative
expression plasmid pET17b (which includes a T7
promoter) was investigated with similar results.

30   It should be noted that the native class II
pilE gene sequence in pPX8002 ends at base 447. The
DNA sequence found downstream (3') from the native
meningococcal classII pilE termination site,
nucleotides 447 to 519, contains an inverted repeat

35   which might form a stem and loop structure. Because

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stem and loop structures can be effective terminators
of transcription, it was postulated that the omission
of this additional 3′ sequence (74 bases) in pPX8002
might affect the transcription of the chimeric class II
5   *pil*E message in *E. coli*. Therefore, all subsequent
cloning restored this downstream 3′ end sequence.

     A systematic replacement of various portions
of the class II *pil*E gene from the *N. meningitidis*
strain with the corresponding regions of the *pil*E gene
10   from *N. gonorrhoeae* strain Pgh3-1 was undertaken in
order to identify regions that inhibited expression.
Replacement regions started at the 5′ or 3′ ends and
were made progressively larger. Double replacements of
5′ and 3′ ends were also constructed until an internal
15   region of only 84 nucleotides from the native *pil*E
class II remained. This region was also replaced,
resulting in the reconstruction of the rGC and, as
expected, this clone expressed rpilin at similar
Coomassie blue stained levels as pPX2000. The
20   following regions (listed by nucleotide numbers) of the
FAM18 *pil*E gene were replaced with the corresponding
regions from Pgh3-1 (listed in parentheses): single
region replacements were 1-108 (1-108), 1-181 (1-181),
1-294 (1-282), 439-499 (478-553), 379-519 (367-553),
25   295-519 (283-553), 295-378 (283-366); double region
replacements were 1-294 (1-282) & 379-519 (367-553), 1-
181 (1-181) & 439-499 (478-553), 1-181 (1-181) & 379-
519 (367-553), 1-294 (1-282) & 439-499 (478-553).

     When these constructs were expressed in *E.
30   coli* TOP10F′ using the pTrcHis expression system, two
constructs produced recombinant protein at levels
detectable by Coomassie blue: the first containing the
replacement of nucleotides 379-519 (which comprises the
disulfide loop and 3′ extension); and the second
35   containing the replacement of both nucleotides 1-181

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(which comprises the conserved 5' region) & 379-519
(which comprises the disulfide loop and 3' extension).
Because replacement of the 5' region alone did not lead
to expression of recombinant protein and because the
first construct retained most of the native
meningococcal pilin sequence, this construct
(nucleotides 379-519) was selected for further
investigation. While the amino acid sequence of the
meningococcaal and gonococcal proteins are
significantly different in this region, it is well
documented (17,18) that the disulfide loop undergoes
significant antigenic variation. Therefore, any immune
response directed against this region (e.g., the
disulfide loop) would exhibit minimal cross-reactivity
among meningococcal strains. Lastly, because the
gonococcal insert is nearly twice the size of the
meningococcal disulfide loop (39 residues versus 18
residues), the resulting chimeric protein migrates on
an SDS-PAGE gel with an apparent molecular weight of
approximately 19,000 daltons.

          The construction of this chimeric gene was
carried out in the following manner. The 5' fragment
was obtained by amplifying pPX8002 (FAM18 class II
pilE) with the following primers: 5' GCG GCC GCC ATG
GAA GCA ATC CAA AAA GGT TTC ACC C 3' (PILE2FWD) (SEQ ID
NO:19) and 5' GCC GCG CGT CTC CGA ACC GGA GTT TTG TTT
GCC 3' (REV-CYS) (SEQ ID NO:20). The gonococcal
disulfide loop (i.e., the 3' end of the gonococcal
gene) was amplified from pPX2000 using primers 5' CCG
GGC CGT CTC GGT TCG GTA AAA TGG TTC TGC 3' (FWD-CYS)
(SEQ ID NO:21) and 5' GGG CCT GGA TCC GTG GGA AAT CAC
TTA CCG 3' (PILEREV) (SEQ ID NO:22). The resulting PCR
products were each purified, digested separately with
restriction enzyme BsmBI, then ligated to form the full
length chimeric pilE, which was amplified using primers

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PILE2FWD and PILEREV. This PCR product was digested
with restriction enzymes NcoI and BamHI, ligated into a
similarly restricted pTrcHisC vector and transformed
into TOP10F' competent cells.

Transformants were cultured and analyzed
using the restriction enzymes NcoI and BamHI. Four
clones with the right sized insert were analyzed with
restriction enzyme StuI. Of these, three provided the
correct restriction map. Two of the three clones with
the correct restriction pattern were sequenced. Clone
#5 had the correct DNA sequence and was designated as
pPX8017. This clone contains the nucleotide sequence
set forth in SEQ ID NO:3, in which nucleotides 1-378
are from N. meningitidis and nucleotides 379-510 are
from N. gonorrhoeae.

Expression was checked with 10mL cultures in
50mL tubes. Cells were grown in SOB supplemented with
100 µg/ml ampicillin and 12 µg/ml tetracycline until
A_{600} approximately 1.0. A culture was induced by the
addition of IPTG to a final concentration of 1 mM.
Growth was allowed to continue for 3-4 hours, at which
point the cells were collected by centrifugation
(13,689 x g for 20 minutes at 4°C) and stored at -20°C.
For the fermentor, an overnight culture from a plate or
frozen stock was used to inoculate a flask containing
500 mL media which was again grown overnight. This
liquid culture was then used to inoculate a Biostat B
Fermentor (Braun Biotech, Allentown, PA) containing 8.9
L of media. Enhanced growth of the bacteria in the
fermentor was obtained when HySoy™-containing media was
supplemented with dextrose at a final concentration of
1% (w/v). When the culture reached A_{600} = 4.0-6.0, IPTG
was added to a final concentration of 1 mM and the
cells were allowed to grow for another 2-4 hours before
being harvested by centrifugation (13,689 x g for 20

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minutes at 4°C). The media was discarded and the cell
pellet stored at -20°C. Upon induction with IPTG,
expression of chimeric class II rpilin protein
increased significantly.

5   Samples of the induced cultures were analyzed
by polyacrylamide gel electrophoresis in the presence
of sodium dodecyl sulfate (SDS-PAGE). The recombinant
meningococcal chimeric class II pilin was visualized
using Coomassie blue staining (apparent molecular
10   weight of approximately 19,000 daltons) and its
identity confirmed by Western blot with a polyclonal
antisera directed against a gonococcal peptide (Glu Ala
Ile Leu Leu Ala Glu Gly Gln Lys Ser Ala Val Thr Glu Tyr
Tyr Leu Asn His Gly Lys) (SEQ ID NO:24), which is
15   located in the conserved region of the amino terminus
of the class II pilin protein. The meningococcal
chimeric class II rpilin was 170 amino acids in length
(including the signal) (SEQ ID NO:4), in which amino
acids 1-126 are from *N. meningitidis* and amino acids
20   127-170 are from *N. gonorrhoeae*.

Example 7
Isolation and Purification of
Recombinant Gonococcal Pilin from *E. coli*

25   The following procedure was used to purify
the recombinant gonococcal pilin obtained in Examples 2
and 3 above. This procedure is also used to purify the
meningococcal recombinant pilin obtained in Example 4,
30   and was used initially to purify the meningococcal
chimeric class I rpilin protein obtained in Example 5,
above. Subsequently, the isolation procedure for the
meningococcal chimeric class I rpilin protein was
modified as described in Example 8 below.

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        Sub-cellular fractionation of the *E. coli*
expressing rpilin demonstrated that the protein was
associated with the cellular membranes, most likely the
inner membrane, based on the ability of 1% (v/v)
Triton™ X-100 to solubilize this protein. When an
attempt was made to remove contaminating *E. coli*
proteins in the presence of 0.05-0.1% (v/v) Triton™ X-
100, it was discovered that, below pH 9.5, the rpilin
did not bind consistently to an ion exchange column.
Therefore, the ability of a number of detergents to
selectively solubilize the rpilin protein from *E. coli*
membrane preparations was examined.

        The cell pellet (approx. 5 g wet weight) from
1 L of culture was thawed by adding 30 mL of 10 mM
Hepes (pH 7.2) (Research Organics, Cleveland, OH), 1 mM
EDTA and the cells broken using a Microfluidizer cell
homogenizer (Microfluidics International Corp., Newton,
MA). The lysate was clarified by centrifugation
(12,000 x g for 10 minutes) and the membranes pelleted
(288,652 x g for one hour). The membranes were
resuspended in 33 mL of 10 mM Hepes (pH 7.4), 1 mM
MgCl$_2$ and extracted with one of the following
detergents: (a) Triton™ X-100 (TX100) (Calbiochem-
Novabiochem International, San Diego, CA), (b) reduced
Triton™ X-100 (Calbiochem), (c) octyl-β-D-
glucopyranoside (OG) (Calbiochem), (d) Zwittergent™ 3-
8 (Z3-8) (Calbiochem), (e) Zwittergent™ 3-10 (Z3-10)
(Calbiochem), (f) Zwittergent™ 3-12 (Z3-12)
(Calbiochem), (g) Zwittergent™ 3-14 (Z3-14)
(Calbiochem), (h) Empigen BB™ (Calbiochem) or (i)
Tween™ 80 (ICN, Cleveland, OH) for one hour at room
temperature.

        Empigen™ BB (1% v/v), Zwittergent™ 3-10 (1%
w/v), reduced Triton™ X-100 (1% v/v), octyl glucoside
(1% w/v) with Zwittergent™ 3-10 (1% w/v) or 3-14 (0.1%

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w/v) each selectively extracted the recombinant pilin
protein with minimal contamination with the *E. coli*
proteins. Zwittergent™ 3-12, even at 0.1% (w/v),
solubilized both the recombinant protein and a
significant number of *E. coli* proteins. Tween™ 80 did
not extract the recombinant protein at any tested
concentration (0.1-1% v/v).

The solubilized proteins were separated from
insoluble membrane material by centrifugation (288,652
x g for one hour). The supernatant (containing rpilin)
was dialyzed overnight at 4°C against 10 mM Tris™ (pH
8.5) containing one of the following non-ionic
detergents: (a) 0.1% (w/v) Zwittergent™ 3-14, (b) 1%
(w/v) Zwittergent™ 3-10 or (c) 1% (w/v) OG. The
dialyzed material was fractionated on a Fractogel™ EMD
TMAE-650(S) (EM Separations Technology, Wakefield, RI)
column equilibrated in 10 mM Tris™ (pH 8.5) and the
respective detergent. The bound protein was eluted
with a linear gradient of 0 to 0.2 M NaCl in 10 mM
Tris™ (pH 8.5) containing the appropriate detergent.
Fractions containing rpilin were pooled, analyzed for
purity and protein content. Occasionally, to increase
the purity of the rpilin, the pooled material was
dialyzed against the starting buffer and fractionated a
second time on the TMAE column.

The rpilin, which was selectively eluted from
the column, was highly purified, as judged by laser
densitometric analysis of a Coomassie blue stained SDS-
PAGE (>90% homogeneous). Similar results were obtained
when the extraction and column chromatography were done
with 1% (w/v) Zwittergent™ 3-10, 1% (w/v) OG or 0.1%
(w/v) Zwittergent™ 3-14. The yield of rpilin, which
was a significant proportion of the total *E. coli*
protein, was approximately 10 mg/L of culture grown in
1.5 L shake flasks with SOB media. When the

recombinant *E. coli* (containing pPX2002) were grown in
a fermentor using HySoy™ based media, the yield of
purified rpilin increased to approximately 30 mg/L of
culture, which corresponds to seven mg rpilin per gram
of cell mass. When 1% dextrose was included in the
fermentor, the overall yield of rpilin increased to
approximately 100 mg/L.

        The purified rpilin was dialyzed against 10
mM sodium phosphate, 140 mM NaCl (pH 7) (PBS)
containing 0.05% (w/v) Z3-14, sterile filtered and
stored at 4°C or frozen at -20°C.

                        Example 8
            Isolation and Purification of Meningococcal
                Chimeric Class I rpilin from *E. coli*

        Large scale cultures of *E. coli* cells
containing pPX2004 were grown in a Biostat B Fermentor
as described in Example 2. Bacterial cells
(approximately 88 grams wet weight of *E. coli* pPX2004)
were resuspended in 440 mL of 10 mM Hepes, 1 mM EDTA
(pH 7.5) and disrupted using a Microfluidizer Model
110Y (Microfluidics Corp., Newton, MA). The disrupted
cells were clarified by centrifugation at 6,084 x g for
20 minutes at 10°C. The supernatant was collected and
the membrane fraction isolated by centrifugation at
205,471 x g for 1 hour at 10°C. The pellet was
resuspended by homogenization in 220 mL of 10mM Hepes,
1 mM MgCl$_2$, 1% (w/v) octyl-β-D-glucopyranoside (pH 7.5)
and stirred for 90 minutes at room temperature. The
suspension was centrifuged at 205,471 x g for one hour
at 10°C. Following centrifugation, the supernatant,
which contained the solubilized chimeric class I
rpilin, was filtered through a 0.22μ Nalgene vacuum
filter and stored at 4°C. The pH of the octylglucoside

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extract was adjusted to pH 8.5 with concentrated NaOH
and subsequently loaded onto a 200 mL TMAE Fractogel™
column (EM Separations Technology, Gibbstown, NJ)
equilibrated with 25 mM Tris™, 0.1% (w/v) Zwittergent™

5   3-14 (pH 8.5). Unbound protein was washed through the
column with an additional 400 mL of the equilibration
buffer. The rpilin was eluted using a linear NaCl
gradient (0-0.2 M NaCl) in 25 mM Tris™, 0.1% (w/v)
Zwittergent™ 3-14 (pH 8.5) over 10 column volumes at a

10   flow rate of 10.0 mL/minute. Fractions containing the
chimeric class I rpilin were pooled and diluted 1:1
with dH$_2$O and loaded onto a 100 mL 40 μm ceramic
hydroxyapatite column (Bio-Rad, Hercules, CA)
equilibrated with 10 mM NaPO$_4$, 0.1% (w/v) Zwittergent™

15   3-14 (pH 6). Unbound protein was washed through the
column with an additional 200 mL of equilibration
buffer. The chimeric class I rpilin was eluted using a
linear NaPO$_4$ gradient (10-150 mM NaPO4) containing 0.1%
(w/v) Zwittergent™ 3-14 over 10 column volumes at a

20   flow rate of 5.0 mL/minute. Fractions were screened by
SDS-PAGE analysis and those containing the chimeric
class I rpilin were pooled. The purified material was
at least 95% pure, as determined by laser densitometry
of Coomassie blue-stained gels. The yield of purified

25   chimeric class I rpilin was approximately 35 mg/g wet
weight cells.

Example 9
Isolation and Purification of Meningococcal
30   ### Chimeric Class II rpilin from _E. coli_

All steps were performed at room temperature
unless specified. Frozen _E. coli_ cells were
resuspended in 10 ml of 10 mM Hepes (pH 7.2), 1 mM EDTA

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per gram of cells and homogenized using a
Microfluidizer cell homogenizer to disrupt the cells.
The cell lysate was clarified by centrifugation at
13,689 x g for 30 minutes. The resulting supernantant
was then centrifuged at 388,024 x g for 30 minutes at
4°C. The supernatant was discarded and the pellet
containing the membranes was frozen at -20°C overnight.
The membrane pellet was resuspended in 9 mL/tube of 10
mM Hepes (pH 7.2), 1 mM MgCl$_2$ and extracted with 1%
(w/v) Zwittergent™ 3-16 (Calbiochem) for one hour.
The suspension was centrifuged at 388,024 x g for 30
minutes and the resulting pellet was extracted again
with Zwittergent™ 3-16 as described above. Following
centrifugation (388,024 x g for 30 minutes), the pellet
was resuspended into 9 mL of 50 mM Tris™ (pH 8.0), 5
mM EDTA and extracted with 1% (w/v) N-laurylsarcosyl
(Sigma) with gentle agitation overnight at room
temperature. This resulted in the solubilzation of the
meningococcal chimeric class II rpilin. The insoluble
material was removed by centrifugation (388,024 x g for
30 minutes) and discarded. Zwittergent™ 3-14 was
added to the supernantant, which contained the
meningococcal chimeric class II rpilin, to a final
concentration of 1% (w/v) and the material was dialyzed
overnight against 50 mM Tris™ (pH 8.0), 10 mM EDTA, 1%
Zwittergent™ 3-14. An aliquot (1 mL, 1.3mg protein)
of the dialyzed material was then passed over a Mono-
Q™ (Pharmacia, Piscataway, NJ) column (5x10 mm) which
was equilibrated in 50 mM Tris™ (pH 8.0), 10 mM EDTA,
1% (w/v) Zwittergent™ 3-14 at a flow rate of 0.5
mL/minute. The unbound material containing the
chimeric class II rpilin was pooled and dialyzed
overnight against 10 mM NaPO$_4$ (pH 6.8), 1% (w/v)
Zwittergent™ 3-14. This material was approximately

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80% pure and was used in the studies described in
Examples 26 and 27 below. Further purification of this
material was obtained by passing it over a 1 mL
hydroxyapatite column (Bio-Rad) which was equilibrated
in 10 mM NaPO₄ (pH 6.8), 1% (w/v) Zwittergent™ 3-14.
The purified meningococcal chimeric class II protein
was eluted with a linear gradient of 0-0.5M NaPO₄
containing 1% (w/v) Zwittergent™ 3-14. Fractions were
screened for chimeric class II rpilin by SDS-PAGE,
using gels which were stained with either Coomassie
blue or silver. Both analyses demonstrated the
presence of a single polypeptide band which had a
molecular weight of approximately 19,000 daltons. This
material was shown to be greater than 95% pure by laser
densitometric analysis of the polyacrylamide gels.

Example 10
Analytical Methods for Gonococcal rpilin

Protein content was determined by the
bicinchoninic acid protein assay (Pierce, Rockford, IL)
using BSA as the standard. The purity of protein
preparations was determined by Coomassie brilliant blue
stained polyacrylamide gel electrophoresis in the
presence of SDS (SDS-PAGE) and analyzed by laser
denistometry with a Personal Densitometer SI (Molecular
Devices). The identity of pilin in the preparations
was confirmed by Western blotting using the monoclonal
antibody described in Example 2, which is raised
against purified pili from *N. gonorrhoeae* strain P9
(Biospacific). The N-terminal sequences of the pilin
proteins were determined using an Applied Biosystems
477A Protein Sequencer. Two sequences were often
detected when the purified rpilin was submitted for N-
terminal sequencing. The major sequence represented

the complete pilin protein, including the seven amino
acid leader sequence. The minor sequence, comprising
10-20% of the sample, was rpilin protein in which the
leader sequence was missing and the sequence started at
5   phenylalanine, the N-terminal residue of the mature
gonococcal pilin protein. For both rpilin protein
forms, sequencing of the amino-terminal residues gave
results which agreed with the sequence deduced from the
DNA sequence.

10   The mass of recombinant pilin was determined
by matrix assisted laser desorption ionization time of
flight (MALDI-TOF) mass spectrometry using a Finnagan
MAT Lasermat™ 2000 (San Jose, CA). The instrument was
calibrated with horse myoglobin to within 0.01% of its
15   expected mass of 16,951.5 daltons. Recombinant pilin
was mixed with an equal volume of a cyano-4-
hydroxycinnamic acid matrix (10 mg/mL in 70:30
acetonitrile : 0.1% (v/v) trifluoroacetic acid/water).
An aliquot (1 μL) of this mixture was deposited on a
20   sample stage, allowed to air dry, and subjected to
MALDI-TOF mass spectrometry analysis. Data from 15
runs (each run representing a sum of 10 shots) were
averaged to determine the mass of rpilin. The
molecular weight of the rpilin (with signal) was
25   determined to 18,001 daltons, which compares well to
the predicted mass of 17,981 daltons based on the amino
acid content. A minor peak with a mass of 17,232
daltons (average) was detected in each lot. The
difference in molecular weights of the two forms of the
30   recombinant pilin (769 daltons) is ascribed to the loss
of the first six amino acids of the leader sequence
(Met Asp Thr Leu Gln Lys) (SEQ ID NO:2, amino acids 1-
6) which has a mass of 774 daltons.

            A very different apparent molecular weight of
35   the rpilin was obtained by size exclusion column

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chromatography using an analytical Superose™ 12 column
(Pharmacia, Piscataway, NJ) equilibrated in PBS
containing 0.05% (w/v) Zwittergent™ 3-14. Under these
conditions, the protein eluted at a position
5   corresponding to a molecular weight of 68,899. This
suggested that the recombinant protein aggregated.
However, the elution of a protein from a size exclusion
column can be greatly influenced by the shape of the
protein. Results from velocity sedimentation
10   centrifugation experiments demonstrated that the
recombinant pilin had a molecular weight in solution of
approximately 45,000 daltons. In an attempt to remove
the detergent (Zwittergent™ 3-14), the recombinant
protein was dialyzed extensively against PBS alone.
15   The dialyzed recombinant protein appeared soluble and
was not pelleted by high speed centrifugation (122,000
x g for one hour). No attempt was made to verify the
complete removal of the detergent from the recombinant
protein. Analysis of this material by gel filtration
20   in PBS indicated that the protein had an apparent
molecular weight of 452,349 daltons. This suggested
that it had undergone further aggregation. The number
of subunits in either aggregate has not been
determined.
25   The value of 452,349 must be considered an
estimate, because the protein may still be in a
micelle, as it is unknown if the detergent was
completely removed from the sample. Given the fact
that rpilin is diluted approximately 15-30 fold when
30   formulated as a vaccine, it appears likely that the
rpilin in the vaccine will have an apparent molecular
weight of approximately 450 kD.

35

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Example 11
Preparation of Immune Sera from rpilin

        Immunogenicity studies were performed using
guinea pigs (female, 200 g) immunized subcutaneously
(s.c.) with 20 μg of purified gonococcal rpilin protein
mixed with an adjuvant. The adjuvants studied were:
(a) Stimulon™ QS-21 (25 μg/dose) in PBS (pH 6); (b)
aluminum phosphate (Lederle Laboratories, Pearl River,
NY, 100 μg/dose) in PBS (pH 7); or (c) PBS (pH 7) only.
Initially, the animals were immunized on weeks 0, 4 and
8 and sera were obtained on weeks 0, 4, 6 and 10.
Analysis of the time course of the immune response
demonstrated that giving a third vaccination at week 8
did not boost the immune response and, therefore, later
studies with the recombinant pilin were terminated at
the week 6 bleed.

        In order to investigate the ability of
adjuvants to modulate the immune response of gonococcal
rpilin, mice (female, 8 weeks old, 5 or 10 animals per
group) were immunized subcutaneously with 1-10 μg of
purified protein on weeks 0, 4 and 6 and sera were
obtained on weeks 0, 4, 6 and 8. Vaginal lavages were
done at week 8 by instilling RPMI 1640 (75 μL) into the
vagina and aspirating 3-4 times. The lavage fluids
from each group were pooled together and 50 μL of fetal
bovine serum was added to each pool.

        For the meningococcal chimeric class I rpilin
protein, the mice received immunizations on week 0 and
4 only and sera was obtained on weeks 0, 4 and 6. For
all rpilin parenteral immunogenicity studies in mice,
the following adjuvants were studied: (a) Stimulon™
QS-21 (25 μg/dose) in PBS (pH 6); (b) aluminum
phosphate (100 μg/dose) in PBS (pH 7); (c) MPL™ (50
μg/dose) in PBS (pH 7); (d) aluminum phosphate (100

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μg/dose) and MPL™ (50 μg/dose) in PBS (pH 7); or PBS
(pH 7) only.

        For the meningococcal chimeric class II
rpilin protein, the guinea pigs received immunizations
of 20 μg protein adjuvanted with Stimulon™ QS-21 (25
μg/dose) in PBS (pH 6) on week 0 and 4 only and sera
was obtained on weeks 0, 4 and 6.

        The ability of the recombinantly expressed
pilins (either gonococcal or meningococcal chimeric
class I) to induce a mucosal immune response was
assessed by immunizing mice intranasally with (a) 1 or
10 μg of gonococcal rpilin in 2.5 μL of saline with or
without 1 μg of native cholera toxin, or (b) 5 μg of
chimeric class I rpilin diluted in 10 μL of PBS (pH 7),
with or without 1 μg of mutant CT-CRM, E29H cholera
toxin. The immunizations were given on weeks 0, 2 and
3.

                    Example 12
            Western Blot Analysis of the Immune
            Response Against Gonococcal rpilin

        The purified gonococcal rpilin was used to
immunize guinea pigs following the protocol described
in Example 11. The antisera derived from guinea pigs
immunized with gonococcal rpilin were analyzed first by
Western blots (data not shown). These blots
demonstrated that the antisera against gonococcal
rpilin recognized a band corresponding to pilin in
whole cell lysate from piliated gonococcal cells; there
was no staining seen in non-piliated cell lysate from
the same gonococcal strain.

        Comparative data were obtained from antisera
from guinea pigs immunized with the gonococcal pilin
oligomer. Pilin oligomer was obtained by dissociation

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of intact pili as previously described (4). Briefly,
this involved dialysis of intact pili against 37 mM
sodium phosphate (pH 12) for 48 hours at 4°C, followed
by dialysis against 50 mM Tris™, 145 mM NaCl (pH 8.0).
The pilin oligomers were then clarified by
centrifugation (100,000 x g for one hour). Following
centrifugation, the pilin oligomers remained in the
supernatant. In comparison to antisera against
gonococcal rpilin, pilin oligomer antisera, while
binding to pilin in the piliated cell lysate, also
bound to a number of other bands in the lysates from
both piliated and non-piliated cells (data not shown).
These bands represent contaminants in the pilin
oligomer preparation and are presumed to be not
associated with pili.

Example 13
Recombinant Gonococcal Pilin ELISA

The endpoint titers against purified proteins
or bacterial cells were determined by ELISA. In all
ELISA procedures, incubations were for one hour at room
temperature, unless otherwise specified. Endpoint
titers were defined as the extrapolated dilution at
which the optical absorbance was 0.10 greater than that
of the blank wells (which do not contain primary
antibody). For the analysis of guinea pig antisera,
purified recombinant pilin was diluted in 0.1 M Tris™
(pH 8) to a final concentration of 1 μg/mL. Aliquots
(100 μL) were added to the wells of a microtiter plate
(Immulon II, Nunc, Naperville IL) and incubated
overnight at 4°C. The plates were washed five times
with PBS containing 0.05% (v/v) Tween™-20 (PBS-T)
using a Skanwash 300 plate washer (Skatron Instruments,
Alexandria, VA). The wells were blocked using 200 μL

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of 1% (w/v) BSA in PBS-T, washed and aliquots of
antisera (diluted in 0.1% (w/v) BSA in PBS-T) were
added to the wells. The plate was then washed and the
bound primary antibodies were detected using 100 μL of
alkaline phosphatase conjugated to rabbit anti-guinea
pig IgG (heavy & light chains) (Zymed Laboratories,
South San Francisco, CA) diluted 1:2000 dilution in
0.1% (w/v) BSA in 50 mM Tris™ (pH 8). The plates were
washed and the color developed using 100 μL per well of
p-nitrophenol phosphate (Sigma) (2 mg/mL in 0.5 M
diethanolamine, 0.25 mM $MgCl_2$, pH 9.8). After 30
minutes, the reaction was stopped by adding 50 μL of 3
N NaOH. The absorbance was read in a Thermomax ELISA
plate reader (Molecular Devices, Sunnyvale, CA) at 405
nm.

All the animals immunized with rpilin
responded very well, as demonstrated by the antigen
ELISAs shown in Table 1.

Table 1

Endpoint Titers for the Binding of Pooled Guinea Pig
Antisera Against Gonococcal rpilin to Purified
Recombinant Gonococcal Pilin Protein*

Prep	Immunogen	Endpoint Titers		
		Week 0	Week 4	Week 6
1	r Pgh3-1 pilin (Prep 1)	≤100	51,345	494,805
2	r Pgh3-1 pilin (Prep 2)	54	30,237	594,298
3	r Pgh3-1 pilin (Prep 3)	≤100	24,830	546,682

*   Three different lots of rpilin were used as
immunogen. Guinea pigs were immunized (s.c.) on weeks 0

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and 4 and bled on weeks 0, 4 and 6. Analyses were done
on pooled sera.

Effect of Adjuvants on Immune Response

5   The effect of the following adjuvants upon
the immune response against gonococcal rpilin was
studied in mice: (a) Stimulon™ QS-21 in PBS (pH 6);
(b) aluminum phosphate in PBS (pH 7); (c) MPL™ in PBS
(pH 7); (d) aluminum phosphate and MPL™ in PBS (pH 7);
10   or PBS (pH 7) only. For the analysis of the mouse
antisera, the antigen ELISA protocol was modified as
follows. The microtiter plates (Costar EIA/RIA,
Corning Costar, Cambridge, MA) were coated with 100 μL
of 1 μg/mL rpilin in PBS overnight at 37°C. The plate
15   was washed five times using PBS containing 0.1% (v/v)
Tween™-20 using a Skantron 300 plate washer. The
wells were blocked with PBS containing 0.1% (w/v)
gelatin and 0.02% (w/v) NaN$_3$. The primary antibody was
diluted in PBS containing 0.1% (w/v) gelatin, 0.05%
20   (v/v) Tween™-20 (PBS-TG) and 0.02% (w/v) NaN$_3$ and 100
μL aliquots were incubated in the microtiter plate for
2 hours. After washing, the bound primary antibody was
detected using biotinylated rabbit anti-mouse IgG (Fc
region) (Brookwood Biomedical, Birmingham, AL) diluted
25   1:8000 in PBS-TG and 0.02% (w/v) NaN$_3$. The plate was
washed and the secondary antibody was detected, in
turn, using streptavidin conjugated horseradish
peroxidase diluted 1:5000 (Zymed Laboratories) in PBS-
TG and 0.02% (w/v) NaN$_3$ (30 minute incubation). The
30   plate was washed and the color was developed using 0.5
mg/mL 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic
acid) (ABTS) in 0.1 M citrate (pH 4.2) containing 0.03%
(v/v) hydrogen peroxide for 30 minutes and monitored at
405 nm using an SLT 340 ATTC microplate reader (SLT
35   Labinstruments, Research Triangle Park, NC). The data

were plotted using a log-log plot and the endpoint
titers were determined as previously described.

When the rpilin was adjuvanted with MPL™, a
humoral immune response in mice was obtained, which was
similar in magnitude to that seen with Stimulon™ QS-21
(Table 2A). Analysis of vaginal washes from the same
animals also revealed a demonstrable IgG titer in the
vaginal washes of these animals (Table 2B).

Table 2A

Effect of adjuvants on the immune response to gonococcal rpilin*

Humoral:

| Adjuvant | Antigen ELISA | Whole Cell ELISA | | | | |
| | rpilin | Homologous Strain | Heterologous Strains | | | |
		Pgh3-1	LB2	#11	T-1	I756
AlPO₄	361,260	249,410	93,444	141,622	42,535	89,615
MPL™	1,262,735	902,891	499,223	586,642	165,301	321,340
AlPO₄ + MPL™	1,159,670	265,269	112,856	160,814	41,368	110,162
Stimulon™ QS-21	4,915,200	1,053,446	478,338	440,014	111,753	333,341

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Table 2B

Effect of adjuvants on the immune response to gonococcal rpilin*

Vaginal Lavages**:

Adjuvant	rpilin Titers		Whole Cell Titers***	
	IgG	IgA	LB2 P+	LB2 P-
AlPO₄	1,452	262	68	≤50
MPL™	6,409	26	1,128	0
AlPO₄ + MPL™	938	12	120	≤50
Stimulon™ QS-21	2,716	175	311	37

* Balb/c mice (5 per group) were immunized with 10 µg of rpilin adjuvanted as described above on week 0, 4 and 6. The animals were bled on weeks 0, 4, 6 and 8. Analyses were done on pooled sera from week 8. Endpoint titers for week 0 were ≤ 5,000.

** Pooled vaginal lavages were obtained on week 8.

*** Whole cell ELISA using dried cells.

P+: piliated cells. P-: non-piliated variants derived from piliated parental cells.

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Example 14
Gonococcal Whole Cell ELISA

The expression by the rpilin of a significant
number of the cross-reactive epitopes found on the
intact, assembled pili was subsequently verified in the
whole cell ELISA, where antisera against purified
rpilin exhibited high titers against numerous
gonococcal strains expressing heterologous pili. The
ability of guinea pig antisera to bind to live
gonococcal cells was done using the following protocol.
Microtiter plates (Immunolon II) were incubated
overnight with 100 μL of 0.01% (w/v) poly-L-lysine
(Sigma) in PBS at 4°C. Overnight agar cultures of
bacteria were harvested into PBS with a Dacron swab and
the turbidity of the suspension adjusted to an A_{600} =
1.0. The poly-lysine solution was discarded from the
microtiter plate and 100 μL aliquots of the bacterial
suspension added to the wells. The plates were washed
five times with PBS using a hand-held Nunc plate washer
and blocked with 200 μL of PBS containing 1% (w/v) BSA
(PBS-B). The plates were then washed five times with
PBS and 100 μL aliquots of antisera diluted in 0.1%
(w/v) BSA in PBS were added to the wells. After
washing five times with PBS, the bound antisera was
detected using 100 μL of the appropriate alkaline
phosphatase conjugates listed above at a 1:2000
dilution in 0.1% (w/v) BSA in PBS. The plates were
washed, the color developed and the absorbance read (at
30 minutes development) as described above.

When analyzed by the whole cell ELISA, the
guinea pig antisera against rpilin bound to piliated
isolates from diverse geographical locations, but not
to the corresponding non-piliated cells of the same
gonococcal strain (Table 3).

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Table 3

Endpoint Titers for the Binding of Guinea Pig Antisera

Against rpilin to Live Gonococcal Cells from Diverse Strains*

Strain	Source	Pgh3-1 rpilin (Prep 1)	Pgh3-1 rpilin (Prep 2)	Pgh3-1 rpilin (Prep 3)
Pgh 3-1 P+	Pittsburgh	2,940,971	2,461,272	2,373,641
Pgh 3-1 P-	Pittsburgh	<250	<250	<250
LB2 P+	Unknown	505,439	481,815	825,517
LB2 P-	Unknown	187	34	114
I-756 P+	Ontario, Canada	689,309	713,904	1,026,231
1948 P+	Romania	290,367	259,487	325,640
1635P P+	Panama	2,714,006	≥546,750	≥546,750
22714FB P+	Fort Bragg, NC	397,763	346,895	498,882
T-1 P+	Unknown	189,490	317,370	272,828
M-2 P+	Unknown	124,906	117,734	151,966
3138K P+	Korea	220,730	179,127	584,044

Table 3 (continued)

Endpoint Titers for the Binding of Guinea Pig Antisera

Against rpilin to Live Gonococcal Cells from Diverse Strains*

Strain	Source	Pgh3-1 rpilin (Prep 1)	Pgh3-1 rpilin (Prep 2)	Pgh3-1 rpilin (Prep 3)
N-2 P+	Norfolk, VA	350,210	337,232	435,164
J474B P+	Jamaica	238,640	205,247	380,970
Fresh Clinical Isolates				
52407 P+	Tampa, FL	598,124	890,678	778,607
#11 P+	Rochester, NY	214,673	292,358	385,985
FA1090:				
2-57-U17 P+	Chapel Hill, NC	1,364,126	2,379,267	3,791,263
2-42-U14 P+	Chapel Hill, NC	1,449,757	1,964,641	11,619,267

*   Three different lots of rpilin were used as immunogen. Guinea pigs were immunized
(s.c.) and bled as previously described. Pooled sera from week 6 bleed were assayed.
Endpoint titers for week 0 sera were ≤250.

P+: piliated cells. P-: non-piliated variants derived from piliated parental cells.

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The whole cell ELISA analysis of mouse
antisera against rpilin (Table 2) was done using
microtiter plates in which the bacteria were dried down
in the wells by the following protocol. Overnight agar
cultures of bacteria were harvested into PBS with a
Dacron swab and the turbidity of the suspension
adjusted to an A_{600} = 0.1. Aliquots (100 μL) of the
bacterial suspension were added to the wells and the
plate was air dried at 37°C. After evaporation of all
the liquid, the plates were sealed and stored at 4°C
until used. The remainder of the assay was done
following the protocol previously described for the
antigen ELISA of mouse antisera. The data from the
whole cell ELISAs (Tables 2 and 3) suggest that rpilin
induces antibodies which bind to conserved epitopes on
the surface of piliated gonococci.

Example 15
Induction of a Mucosal Immune Response
Against Gonococcal rpilin

Because gonorrhea is a disease of the genital
mucosae, it was of interest to examine the ability of
mucosal immunization to induce a mucosal immune
response. This was accomplished in the following
manner. Mice were immunized intranasally with
gonococcal rpilin in saline (1 or 10 μg in 10 μL) with
or without 1 μg of native cholera toxin on weeks 0, 1
and 2. Groups of five Swiss-Webster mice were
immunized intranasally with rpilin with or without
cholera toxin on weeks 0, 1 and 2. Analyses were
conducted on pooled sera. Endpoints titers for week 0
were <50.

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As shown in the following Table 4, there was
a significant immune response detected in the antigen
ELISA when the animals were immunized with rpilin in
the absence of adjuvant (the week 0 titers were <300).
This response was enhanced by the addition of native
cholera toxin.

Table 4

Endpoint Titers for the Binding of Pooled Mouse
Antisera Against Gonococcal rpilin to Purified
Recombinant Gonococcal Pilin Protein

Antigen (dose µg)	Adjuvant (dose µg)	Day 22	Day 36	Day 50
rpilin (1)	none	1,684	1,287	2,291
rpilin (10)	none	29,046	20,658	71,067
rpilin (1)	cholera toxin (1)	4,673	7,526	3,273
rpilin (10)	cholera toxin (1)	107,011	321,714	280,079
None	cholera toxin (1)	<300	<300	<300

Next, these sera were examined for their
ability to bind to intact, piliated gonococcal cells by
an ELISA performed against cells from *N. gonorrhoeae*
strain FA1090. The cells were dried down onto the
microtiter plate as described previously. As shown in
Table 5, a low titer was detected for rpilin alone (the
week 0 titers were <300). The binding to piliated
cells was greatly enhanced by addition of cholera
toxin.

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Table 5

Endpoint Titers for the Binding of Pooled Mouse
Antisera Against Gonococcal rpilin to Whole,
Piliated Gonococcal cells

5

Antigen (dose μg)	Adjuvant (dose μg)	Day 22	Day 36	Day 50
rpilin (1)	none	1,009	852	729
rpilin (10)	none	6,009	6,252	5,564
rpilin (1)	cholera toxin (1)	1,133	1,456	1,048
rpilin (10)	cholera toxin (1)	209,522	57,767	38,127
None	cholera toxin (1)	239	<500	<500

Example 16

Gonococcal Immunoelectron Microscopy

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        Visualization of the binding of antisera to
piliated bacteria was conducted using the following
protocol. Gold coated grids were spotted with an
aliquot from a late log phase liquid culture of recA⁻,

15   piliated N. gonorrhoeae for five minutes and the excess
fluid was removed with a piece of filter paper. The
grids were blocked with PBS-B for five minutes,
followed by 1% (w/v) fish gelatin (Fluka, Ronkonkoma,
NY) in PBS for 10 minutes. The grids were then

20   incubated with polyclonal antisera diluted in PBS-B for
1-60 minutes at room temperature. Unbound antibodies
were removed by floating the grid on droplets of PBS-B
(4 x 30 seconds). The bound primary antibodies were
detected by floating the grid on a drop of 12 nm

25   colloidal gold bound to donkey anti-guinea pig IgG
(Jackson Research Labs, West Grove, PA) diluted 1:5 in
PBS-B for 30 minutes. The grids were then washed five

times on droplets of PBS-B as described above. The
sample was then stabilized with 1% (v/v) glutaraldehyde
(Electron Microscopy Sciences, Fort Washington, PA) in
PBS for three minutes, then rinsed 5 x 1 minute in
distilled water and lightly stained using NanoVan stain
(NanoProbes, Stony Brook, NY) (pH 8) for 30 seconds.
All liquid was removed by touching the grid to a piece
of filter paper and examined on a Zeiss 10C
transmission electron microscope at 15-75,000X using an
acceleration voltage of 80 kv.

        As shown by immunoelectron microscopy (Figure
1A), antibodies against the recombinant pilin were
bound along the length of the heterologous pili
filaments on the surface of gonococci. This suggested
that the antibodies would bind to these epitopes which
would be present on the surface of the bacteria *in
vivo*.

                        Example 17
        Whole Cell ELISA of Gonococcal rpilin Oligomer

        In order to distinguish the biochemical and
immunological properties of rpilin from those of intact
pili (or pilin oligomer), purified rpilin was converted
to rpilin oligomer by dialysis against pH 12 phosphate
buffer. Antisera induced by this material (rpilin
oligomer) was examined for its ability to bind to live,
piliated gonococci using the whole cell ELISA. As
shown in Table 6, antisera against rpilin oligomer had
significantly lower endpoint titers for binding to
diverse piliated gonococcal cells as compared to
antisera induced by untreated rpilin. This suggests
that rpilin oligomer had lost a significant number of
the cross-reactive epitopes normally present on the
rpilin protein.

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Table 6

Effect of pH 12 ("oligomerization") on the Endpoint Titers for rpilin Guinea Pig Antisera Binding to Piliated *N. gonorrhoeae* Cells*

Strain	rpilin	rpilin Oligomer**
I-756	927,564	16,903
FA-19	107,100	1,982
FA1090 (2-57-U17)	721,786	6,737
LB2	905,711	4,205
#11	225,602	6,999
#4	288,120	8,104
3138K	106,315	5,429
T-1	497,987	42,162
1948	166,864	8,616
J474B	576,640	25,002

* Guinea pigs (4 per group) were immunized (s.c.) with 20 μg of rpilin antigen adjuvanted with 25 μg of Stimulon™ QS-21 on weeks 0 and 4. Pooled sera from week 6 were analyzed.

**Purified rpilin was dialyzed against 37 mM sodium phosphate (pH 12) for 48 hours at 4°C, followed by dialysis against PBS for 24 hours.

Example 18
Inhibition of Adherence to Human Cervical Cells

Because pili mediate the initial binding of *N. gonorrhoeae* to human mucosal cells, the ability of rpilin antibodies to inhibit the attachment of these

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bacteria to epithelial cells was investigated. ME-180
cells, which were derived from a cervical carcinoma,
were selected. In addition, to minimize the clumping
of the piliated bacteria in these experiments, they
were grown in liquid suspension cultures. This
required using recA⁻ derivatives of gonococcal strains
Pgh3-1 or I756 in order to maintain pili expression.
These recA⁻ strains did not show significant clumping
during the 4-5 hours growth in liquid culture, which
made it easier to interpret the results.

In these experiments, an eight well chamber
slide (Nunc) was seeded with ME-180 cells such that the
cells were 80-90% confluent on the day of the
experiment. An overnight culture of recA⁻ gonococcal
cells was swabbed into PBS (warmed to 37°C) and used to
inoculate a flask of liquid GC media supplemented with
0.4% (w/v) $NaHCO_3$ to a final A_{600} = 0.2. The cell
culture was incubated with shaking at 37°C at 120 RPM
for approximately four hours, at which point the
culture reached an $A_{600} \cong 0.8$. The bacterial cell
suspension was diluted 1:8 in RPMI 1640. The wells of
a second 8 well chamber were incubated for at least one
hour with 300 μL of RPMI 1640 and fetal calf serum.
The RPMI 1640 block was discarded and 40 μL of antisera
or RPMI 1640 (without calf serum) added, followed by
260 μL of the diluted bacterial suspension ($\approx 8 \times 10^7$ CFU)
and incubated for one hour at 37°C/5% (v/v)CO_2. The
chamber well slide with the ME180 cells was washed once
with RPMI 1640 devoid of antibiotics. Then, pre-
incubated mixtures of bacteria and antisera were added
onto the ME180 cells and the slides incubated for 30
minutes at 37°C. The media containing unbound
bacterial cells was removed from the cervical cell
monolayer and the wells washed gently three times with
RPMI 1640. After the last wash, the chamber wells were

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removed from the slide, the cells fixed in methanol for
30-60 seconds and stained with Wright-Giemsa stain (VWR
Scientific, West Chester, PA). After destaining in
water, coverslips were mounted over each well.

5   The slides were examined by light microscopy
using oil immersion and pictures of representative
views were taken by a person blinded as to the test
sera. The resulting pictures were analyzed by persons
who were also blinded as to the identity of the

10   samples. In addition, because the piliated gonococci
bound the epithelial monolayers in clumps, the effect
of antisera on the binding of the gonococcal cells was
quantitated by counting clumps of bacteria instead of
individual bacteria. The numbers of clumps of piliated

15   bacteria observed in ten random scans across each well
were determined. The percent difference between wells
containing immune and normal sera was determined.
Again, this analysis was done independently by
researchers blinded as to the samples that they were

20   analyzing.

            Initial results demonstrated that piliated
and non-piliated cells had different binding patterns
to the confluent monolayers of ME180 cells. Both
piliated strains of gonococci typically bound as

25   bacterial clumps to selected epithelial cells within
the confluent monolayer. In contrast, the
corresponding isogenic non-piliated bacteria either did
not bind (Pgh3-1) or showed a low level, disperse
binding (I756) to the epithelial monolayer. Thus, the

30   aggregation of the bacteria on the epithelial monolayer
correlated with the presence of pili.

            Next, the ability of guinea pig antisera
against Pgh3-1 rpilin to inhibit the binding of
piliated cells of strain I-756 to ME-180 cells was

35   tested. Analysis of representative pictures (compare

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Figure 2A (week 6) to Figure 2B (week 0)) demonstrated that antibodies against rpilin significantly inhibited the binding of piliated gonococci to cervical epithelial cells. In contrast, the rpilin antisera had
5   no effect on the binding of non-piliated cells from the same strain when compared to normal guinea pig sera (data not shown). While the number of bacteria bound under these conditions could not be determined, adherence was quantitated by counting the bacterial
10 clumps bound in the presence of either normal or immune sera. Using this method, it was determined that antisera against rpilin resulted in ≈60% decrease in the bacterial clumps bound to epithelial cells as compared with normal guinea pig serum.
15   While this assay did not yield readily quantifiable data, the estimates obtained by counting bacterial clumps probably resulted in an underestimation of the effectiveness of the antisera. This is because of the binding mediated by other cell
20 surface components (e.g., Opa proteins) which would not be expected to be overcome with antisera against rpilin alone.

                        Example 19
25   Analytic Methods for Meningococcal
                 Chimeric Class I rpilin

        The analytic methods described in Example 10 were used for the chimeric meningococcal class I
30 rpilin. As determined by MALDI-TOF mass spectroscopy, the subunit molecular weight of the meningococcal chimeric class I rpilin protein is 17,659 daltons, which is very similar to the anticipated mass of 17,676 daltons based on the amino acid content. When this
35 protein was analyzed by size exclusion chromatography

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using a Superose™ 12 column equilibrated in PBS
containing 0.05% (w/v) Zwittergent™ 3-14, the chimeric
class I rpilin has an apparent molecular weight of
69,480 daltons. This is essentially identical to the
apparent molecular weight of gonococcal rpilin (68,899
daltons) analyzed under the same conditions.

The N-terminal sequence of the purified
meningococcal class I rpilin protein was determined by
Edman degradation and the results (from three different
samples) agreed with the predicted protein sequence.
At least 35 residues were determined for all sequences.

Example 20
Meningococcal Chimeric Class I rpilin ELISA

The endpoint titers against purified proteins
or bacterial cells were determined by ELISA using the
methods described in Examples 13 and 14. The ELISAs
were performed using pooled sera from the respective
bleeds. Whole cell ELISA was done on meningococcal
cells which had been heat killed (56°C for 60 minutes)
or dried down directly onto the microtiter plates. The
cell suspension was diluted to an absorbance of 0.1 at
620 nm and 100 μL aliquots were placed into the wells
of microtiter plates. Each plate was dried at 37°C or
at room temperature, sealed and stored at 4°C until
used. The protocol for the whole cell ELISA was
modified as follows: (1) primary and secondary antisera
were diluted in PBS containing 0.1% (v/v) Tween™-20
and 0.1% (w/v) BSA; and (2) the plates were washed five
times with PBS containing 0.05% (v/v) Tween™-20 using
a Skanwash 300 plate washer.

All the guinea pigs immunized with the
chimeric class I rpilin responded very well, as
demonstrated by the antigen ELISAs shown in Table 7.

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Table 7

Endpoint Titers for the Binding of Meningococcal
Chimeric Class I rpilin Antisera to Purified
Meningococcal Chimeric Class I rpilin*

Adjuvant:

Bleed:	Stimulon™ QS-21	AlPO$_4$	None
Week 0	23	12	32
Week 4	26,607	12,067	4,829
Week 6	1,519,956	372,539	302,911

* Guinea pigs (four per group) were immunized (s.c.) on
weeks 0 and 4 with 20 µg of chimeric class I rpilin
adjuvanted with (a) 25 µg of Stimulon™ QS-21 in PBS
(pH6); (b) 100 µg of AlPO$_4$ in saline; or (c) PBS (pH7)
only. The animals were bled on weeks 0, 4 and 6.
Pooled sera was used for all analyses.

Significant responses were also seen with the
ELISAs to piliated cells, as shown in Table 8.

Table 8

Endpoint Titers for the Binding of Meningococcal
Chimeric Class I rpilin Antisera to Piliated Cells from
N. meningitidis (strain H355)*

Adjuvant:

Bleed:	Stimulon™ QS-21	AlPO$_4$	None
Week 0	28	19	53
Week 4	1,293	1,052	381
Week 6	61,497	25,477	16,467

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* Cells were heat killed.

Effects of Adjuvants on Immune Response

        The effect of adjuvants upon the immune
response against the meningococcal chimeric class I
rpilin was studied in mice using the methods described
in Example 13. As shown in Table 9, the most
significant response for the binding of antisera with
the meningococcal chimeric class I rpilin was achieved
with the addition of Stimulon™ QS-21.

Table 9

Endpoint Titers for the Binding of Meningococcal
Chimeric Class I rpilin Antisera to Purified
Meningococcal Chimeric Class I rpilin*

Adjuvant:

Bleed:	Stimulon™ QS-21	AlPO₄	MPL™	AlPO₄/MPL™	None
Week 0	<50	44	<50	31	<50
Week 4	44,011	29,925	40,146	110,093	<250
Week 6	707,084	103,437	284,455	137,686	115,022

* Mice (ten per group) were immunized (s.c.) on weeks 0
and 4 with 10 µg of meningococcal chimeric class I
rpilin adjuvanted with (a) 25 µg of Stimulon™ QS-21 in
PBS (pH 6); (b) 100 µg of AlPO₄ in saline; (c) 50 µg
MPL™ in PBS (pH 7); (d) 100 µg of AlPO₄ and 50 µg MPL™
in saline; or (e) PBS (pH 7) only. The animals were
bled on weeks 0, 4 and 6. Pooled sera was used for all
analyses.

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As shown in Table 10, the most significant response for the binding of antisera to piliated cells from *N. meningitidis* was also achieved with the addition of Stimulon™ QS-21.

Table 10

Endpoint Titers for the Binding of Meningococcal Chimeric Class I rpilin Antisera to Piliated Cells from *N. meningitidis* (strain H355)*

Adjuvant

Bleed:	Stimulon™ QS-21	AlPO$_4$	MPL™	AlPO$_4$/MPL™	None
Week 0	172	146	153	160	<50
Week 4	6,088	2,310	4,205	5,647	311
Week 6	171,718	25,135	52,053	17,039	16,617

* Cells were heat killed.

Further analyses demonstrated that this antisera against the chimeric class I rpilin bound to meningococcal cells which expressed either class I pilin or, in some cases, class II pilin. The results, shown in Table 11, evidence this partial cross-reactivity.

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Endpoint Titers for the Binding of Meningococcal
Chimeric Class I rpilin Antisera to Piliated Cells from
*N. meningitidis**

Adjuvant

Strain	Class Pilin expressed	Day 0	Stimulon™ QS-21	AlPO₄	MPL™
H355	I	409	127,383	41,190	102,987
M982	I	217	>36,540	>36,540	>36,540
CDC1521	II	988	2,602	1,345	1,768
FAM18	II	3,518	>36,540	26,513	>36,540

* Cells were dried down (at room temperature) directly
onto microtiter plates without being heat killed.

Example 21
Meningococcal Chimeric Class I rpilin
Immunoelectron Microscopy

Visualization of the binding of chimeric
meningococcal class I rpilin antisera to piliated cells
from *N. meningitidis* strain H355 was conducted using
transmission-electron microscopy as follows. A colony
from an overnight culture of *N. meningitidis* was
carefully picked up using a sterile loop and placed in
a microfuge tube containing 0.5-1.0 mL of modified
Franz media [1.3 g/L glutamic acid, 20 mg/L cysteine,
10 g/L Na₂HPO₄·7H₂O, 90 mg/L KCl, 6 g/L NaCl, 2 g/L
yeast dialysate and supplemented with dextrose (4 g/L),
glutamic acid (100 mg/L), cocarboxylase (200 μg/L) and
ferric nitrate (5 mg/L)]. Gold coated grids were
spotted with an aliquot of the cell suspension for five

spotted with an aliquot of the cell suspension for five
minutes and the excess fluid was removed with a piece
of filter paper. The bacterial cells were then fixed
with 4% (v/v) paraformaldehyde, 0.1% (v/v)
glutaraldehyde in PBS for 30 minutes at room
temperature. The grids were incubated, in sequence,
with (a) PBS-B for five minutes, (b) 1% (w/v) fish
gelatin in PBS for 10 minutes, and (c) PBS containing
0.2 M glycine for five minutes. The blocked grids were
then probed with antisera against meningococcal
chimeric class I rpilin protein as described in Example
16. As shown in Figure 3A), the antibodies against the
meningococcal chimeric class I rpilin protein bound
along the length of the pili. In contrast, normal
serum (week 0) did not show any binding to the pili
(Figure 3B).

<u>Example 22</u>
Cross-reactivity of Meningococcal Chimeric Class I
<u>rpilin Antisera with Gonococcal Piliated Cells</u>

Based on the sequence similarity of
meningococcal class I pilin and gonococcal pilin, it
was shown in Example 4 above that antisera directed
against gonococcal rpilin recognized and bound to
piliated meningococcal cells. In this example, it is
demonstrated that antisera raised against meningococcal
chimeric class I rpilin binds to piliated gonococcal
cells. The data from the mouse and guinea pig
experiments are summarized in Tables 12 and 13,
respectively.

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Table 12

Endpoint Titers for the Binding of Mouse Antisera
Against Meningococcal Chimeric Class I rpilin to
Piliated *N. gonorrhoeae* Cells

Antigen / Adjuvant

GC Strain	Nm rclass I pilin					GC rpilin + MPL™
	Stimulon™ QS-21	AlPO₄	MPL™	AlPO₄/ MPL™	None	
I756	4,527,943	79,927	114,958	56,627	57,426	356,936
FA1090	531,627	40,406	97,224	31,267	38,122	219,600

Table 13

Endpoint Titers for the Binding of Guinea Pig Antisera
Against Meningococcal Chimeric Class I rpilin to
Piliated *N. gonorrhoeae* Cells

Antigen / Adjuvant

GC Strain	Nm rclass I pilin			GC rpilin + Stimulon™ QS-21
	Stimulon™ QS-21	AlPO₄	None	
I756	301,969	122,714	78,111	46,424
FA1090	322,311	262,422	170,842	108,094

GC - *N. gonorrrhoeae*; Nm - *N. meningitidis*

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Example 23
Passive Protection Against Meningococcal Bacteremia
by Meningococcal Chimeric Class I rpilin Antisera

An accepted animal model for evaluating the
ability of vaccines to protect against meningococcal
bacteremia is the infant rat model originally described
by Saukkonen and Leinonen (34). The ability of guinea
pig antisera against meningococcal chimeric class I
rpilin (adjuvanted with Stimulon™ QS-21) to protect
against bacteremia caused by a meningococcal strain,
which expresses a pilin, was tested. On day 0,
Sprague-Dawley infant rats (4-5 days old) were
passively immunized (i.p.) with 0.1 mL of guinea pig
antiserum (week 6) against chimeric class I rpilin
diluted 1:5, 1:10 or 1:20 in PBS. The control group
received 0.1 mL injection of normal guinea pig serum
(week 0) diluted 1:5 in PBS. Twenty-four hours later,
the animals were challenged i.p. with approximately 5 x
10^5 colony forming units (cfu) in 0.1 mL of piliated *N.
meningitidis* (strain H355). Three hours after
challenge, the animals were sacrificed and aliquots of
cardiac blood were diluted and plated onto GC agar
plates. The plates were then incubated for 18-24 hours
at 37°C with 5% CO_2. The bacterial colonies were
counted and the level of bacteremia was then
determined. One way analysis of variance t test was
used to compare groups receiving immune serum (week 6)
with the control group receiving normal serum (week 0).
As shown in Table 14 below, animals passively immunized
with guinea pig antiserum specific for meningococcal
chimeric class I rpilin showed more than a log
reduction in the level of bacteremia as compared to
those animals immunized with normal guinea pig serum.

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This difference was statistically significant, with a p value of <0.05.

Table 14

Ability of Guinea Pig Antisera Against Meningococcal Chimeric Class I rpilin Protein to Prevent Bacteremia in Infant Rats Challenged with Piliated *N. meningitidis* (Strain H355)*

Bleed	Dilution	Mean cfu ± std
Week 0	1:5	4.87 ± 0.18
Week 6	1:5	3.55 ± 0.48**
Week 6	1:10	3.63 ± 0.36**
Week 6	1:20	3.98 ± 0.75**

* Antisera against meningococcal chimeric class I rpilin protein was obtained from guinea pigs as described in Table 7.

** p <0.05.

cfu ± std = colony forming units ± standard deviation.

Example 24
Induction of a Mucosal Immune Response Against
Meningococcal Chimeric Class I rpilin

Mice were immunized intranasally with meningococcal chimeric class I rpilin in saline (5 µg in 10 µL) with or without 1 µg of a cross-reactive mutant form of cholera toxin (CT-CRM, E29H) on weeks 0, 1 and 2. As shown in the following Table 15, there was a significant immune response detected in the antigen ELISA when the animals were immunized with rpilin in the absence of adjuvant. This response was enhanced by the addition of cholera toxin.

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Table 15

Endpoint Titers for the Binding of Pooled Mouse
Antisera Against Meningococcal Chimeric Class I rpilin
to Purified Recombinant Meningococcal Chimeric Class I
rpilin Protein*

	rpilin, no adjuvant	rpilin plus CT-CRM
Sera		
IgG	6,168	1,181,871
IgA	490	3,940
Bronchial wash**		
IgG	<10	580
IgA	<10	19
Nasal wash**		
IgG	<10	98
IgA	12	236
Vaginal wash**		
IgG	174	70
IgA	15	687

* Pooled sera from week 4 were analyzed. Endpoint
titers for pooled sera from week 0 for IgG and IgA were
<50.

** Washes were performed as follows:

Bronchial: Lungs were washed 5 times with 1 mL
RPMI 1640, then 50 µL fetal bovine serum (FBS) was then
added to the sample, which was clarified by

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centrifugation (12,000 x g x 5 minutes) and stored at -20°C.

Nasal: The nasal passages were washed once with 0.5 mL of RPMI 1640 and 20 μL of FBS was then added to the sample before storage at -20°C.

Vaginal: Vaginas were washed 5 times with 0.075 mL of RPMI 1640 and 10 μL of FBS was then added to the sample before storage at -20°C.

Example 25
Active Protection Against Meningococcal Colonization by Meningococcal Chimeric Class I rpilin Antisera

The initial step in meningococcal disease in human beings is the colonization of the nasopharynx by the bacteria. In this process, pili are believed to play a major role in mediating the inital attachement to the epithelial cells. A number of researchers have described procedures for colonizing the nasophayrnx in neonatal animals, but no one has investigated this as a model for testing the efficacy of meningococcal vaccines (35). In order to assess the invention described herein, a nasal colonization model for *N. meningitidis* using adult outbred mice has been developed. Swiss-Webster mice were immunized with meningococcal chimeric class I rpilin adjuvanted with MPL™ subcutaneously on weeks 0, 4 and 8. At week 10, the animals received an intraperitonal injection of 2 mg iron dextran (Sigma) and were challenged intranasally with approximately 1 x 10⁷ cfu of mid-log phase piliated meningococci in a volume of 10 μL which also contained 40 μg of iron dextran. On day 1 after challenge, half the animals received an additional intraperitonal injection of 2 mg iron dextran. The

number of viable bacteria in the nose were determined
on days 1 and 2 after challenge by plating nasal tissue
homogenates on GC agar plates containing selective
antibiotics. The results are shown in Table 16.

Table 16
Number of Viable Bacteria (cfu) Recovered from Nasal
Homogenates of Mice Challenged with Piliated *N.
meningitidis* Strain H355*

Antigen (Dose μg)*	cfu per nose	
	Day 1**	Day 2**
H355 Whole cell (25)	1,165	67
Class I rpilin (10)	6,866	63
Saline	17,943	3,406

* All vaccines were formulated with 100 μg of MPL™
per dose. Each group consisted of five mice.
** Days after intranasal challenge.

Example 26
Western Blot Analysis of the Immune
Response Against Meningococcal Class II Chimeric rpilin

The purified meningococcal class II chimeric
rpilin was used to immunize guinea pigs following the
protocol described in Example 11. The antisera derived
from guinea pigs immunized with meningococcal class II
chimeric rpilin were analyzed first by Western blots
(data not shown). These blots demonstrated that the
antisera against meningococcal class II chimeric rpilin
recognized a band corresponding to pilin in whole cell
lysate from piliated meningococcal cells which
expressed either class II pilin (FAM18) or class I
pilin (H355). In contrast, antisera directed against

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an extract from *E. coli* containing the pTrcHis vector
only did not react with either pilin band in these
Western blots.

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Example 27
Binding of Antisera against Meningococcal Chimeric
Class II rpilin to Piliated Meningococcal Cells

Antisera elicted against partially purified
10   meningococcal chimeric class II rpilin was shown to
bind to meningococcal cells from the homologous strain,
FAM18 with a titer of >36,450 (the week 0 titer was
473).

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